Nucleic acid based amplification methods for the detection, quantification and characterization of viruses in clinical specimens

THESIS

Submitted in partial fulfillment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

by

SHYAMALA GANESAN

Under the supervision of

Dr. H. N. MADHAVAN

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI (RAJASTHAN) INDIA

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CERTIFICATE

This is to certify that the thesis entitled "Nucleic acid based amplification methods for the detection, quantification and characterization of viruses in clinical specimens" and submitted by Shyamala Ganesan, ID No. 2003PHXF430 for the award of Ph.D Degree of the institute, embodies the original work done by her under my supervision.

Signature in full
of the supervisior:

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ABSTRACT

Aim:

The aim of the present study is to identify the nature of genotypes and serotypes of viruses causing congenital cataract and epidemic conjunctivitis in Indian patients which may be different from those reported from other parts of the world.

Methods:

Nucleic acid based molecular biological methods like uniplex Polymerase chain reaction; semi-nested PCR, nested PCR, uniplex Reverse Transcriptase Polymerase chain reaction and nested RT-PCR were applied on various clinical specimens for the detection of various viral etiological agents causing congenital cataract and epidemic conjunctivitis. Quantification of the Herpes simplex viral particles were done by the application of standardized Real Time PCR. The genotype identity was established by DNA sequencing and by analyzing the sequences using MultAlin software. The serotype of HSV was confirmed using various techniques like PCR based Restriction Fragment Length Polymorphism (PCR - RFLP) and DNA sequencing. Conventional methods of Indirect Immunofluorescence staining (IIF) and virus isolation in cell cultures were also attempted.

Results:

The results of the present study indicated the association of Rubella virus (RV) with 12% of congenital cataract patients in less than one-year age. Twenty RV isolates have been obtained using the conventional cell culture methods. 18 of the twenty isolates have been genotyped as belonging to RGI and two to RGII. Direct evidences of association of RV and HSV 2 were demonstrated in 18 (36 %) lens aspirates with RV in 9 (18 %) and HSV 2 in 9 (18 %) others. VZV was not detected in any of the lens aspirates tested. The results of PCR-RFLP and DNA sequencing for HSV serotype confirmation were in concordance with that of the snPCR. Conjunctival swabs collected from patients of Epidemic conjunctivitis indicated the presence of Adenovirus (Ad) in 15% of the patients and remaining 77.0% of the patients were positive for Enterovirus (EV). 49% of these patients turned positive for Coxsackie A24 variant (CA24v) and DNA sequencing indicated 98% homology with that of the CA24v.

Conclusion:

For the first time in India, such direct evidences of these virus associations with congenital cataract have been demonstrated. Detection of HSV 2 DNA in as many number of lens aspirates as that of RV indicated its important role in the causation of

congenital cataract in patients attending our hospital Hence newer serotypes namely HSV2 and RGI are the main etiological agents in the causation of the disease in Indian Population and Coxsackie A24variant has caused the epidemic of acute hemorrhagic conjunctivitis in the south Indian population during June to September 2006.

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LIST OF ABBREVIATIONS / SYMBOLS

%	Percentage
^{o}C	Degree Celsius
AHC	Acute Hemorrhagic Conjunctivitis
ARN	Acute Retinal Necrosis
Ad	Adenovirus
AH	Aqueous Humor
BLAST	Basic Local Algorithm Search Tool
BPB	Bromophenol Blue
CO_2	Carbon – di- Oxide
CNS	Central Nervous system
CSF	Cerebro Spinal fluid
CFT	Complement Fixation Test
CFA	Complement Fixing antigen
CRS	Congenital Rubella Syndrome
CS	Conjunctival swab
CMV	Cytomegalovirus
CID	Cytomegalic Inclusion Disease
CPE	Cytopathic effect
CA24v	Coxsackie A24 variant
ddNTPs	Di deoxynucleotide Triphosphates
dNTPs	Deoxynucleotide Triphosphates

DNA	Deoxy Ribonucleic acid
DMEM	Dulbeccos' Minimum Essential Medium
EV 70	Enterovirus 70
EIA	Enzyme Immunoassay
EKC	Epidemic Keratoconjunctivitis
EDTA	Ethylene di amine tetra acetic acid
FBS	Fetal Bovine Serum
GS	Genital swab
GI	Genotype I
GII	Genotype II
gD	Glycoprotein D gene
GM	Growth Medium
GTC	Guanidium Thiocyanate
HSV	Herpes simplex virus
HSV 1	Herpes simplex virus type 1
HSV 2	Herpes simplex virus type 2
HeLa	Human carcinoma of cervix cell line
HEP-2	Human Epithelioma of larynx cell line
IIF	Indirect Immunofluorescence staining
LA	Lens aspirate
3%MM	Maintenance Medium with 3% fetal bovine serum
MEM	Minimum Essential Medium
mtrII	Morphological Transforming Region II

Mult- Align	Multiple Sequence aligment
NCBI	National Centre for Biotechnology
NFATCC	National Facility for Animal Tissue Culture
NIV	National Institute of Virology
nPCR	Nested Polymerase Chain reaction
nRT-PCR	Nested Reverse Transcriptase Polymerase chain reaction
NT	Neutralization test
OD	Optical Density
PHA	Passive Hemagglutination
PCR	Polymerase Chain Reaction
PCR-RFLP	Polymerase chain reaction based Restriction Fragment Length Polymorphism
RT	Real Time
RE	Restriction Enzyme
RT-PCR	Reverse Transcriptase Polymerase Chain reaction
RNA	Ribonucleic acid
RV	Rubella Virus
sn-PCR	Semi- nested Polymerase chain reaction
SIRC	Statens Serum Institute Rabbit Corneal epithelial cell line
TC	Tissue culture
TBE	Tris Boricacid EDTA
UV	Ultra violet
uPCR	Uniplex Polymerase chain reaction

uRT-PCR	Uniplex Reverse Transcriptase Polymerase chain reaction
Vero	Veneral Enteric Research Organization
VMK	Vervet Monkey Kidney
VR	Viral Retinitis
VF	Vitreous fluid

Shyamala Ganesan – Brief Biography

She is a Research Fellow at the L & T Microbiology Research Centre, Sankara Nethralaya, Chennai. She did Master of Science in Medical Laboratory Technology (2003) from Birla Institute of Technology and Science, Pilani, Rajasthan and registered for PhD in 2004 from the same institute. She is a member of Indian Association of Medical Microbiologists. She got fellowship from Indian Council Medical Research (ICMR), India (2004 – 2006). Her paper published in Indian Journal of Medical Microbiology won the Best Published Paper in Virology for the year 2006. Her knowledge and skill are in Nucleic acid based molecular biological techniques and tissue culture techniques. She has published 5 papers (in Indian Journal of Medical Research, Indian Journal of Pathology & Microbiology, Indian Journal of Medical Microbiology).

Dr. H. N. Madhavan – Brief Biography

He is the Director of Research and Professor of Microbiology & Head of L & T Microbiology Research Centre. He did MBBS (1957) from University of Madras, India; MD (1963) from Andhra University, India and Ph. D (1980) from University of Madras, India. He is a Fellow of Royal Society of Tropical Medicine & Hygiene., UK; Member of Association for Research in Vision and Ophthalmology, USA; Member of American Association for the Advancement of Science., USA; Fellow of the Academy of Medical Sciences (India); Founder Member of Indian Association of Medical Microbiologists.; Member of Indian Association of Pathologists and Microbiologists.; Fellow of the Indian College of Pathologists; Life Member of Indian Virological Society. Recipient of several awards: W.H.O. Fellowship in virology during 1970-71 Dr.D.R.Varman Endowment Oration Award for the year l994; "Swarna Latha Punshi" award as "Best Research Worker" for the year 1994; Dr. K.C. Basu Mallik Award for the Best Research Work 1995; The IAMM Endowment Award - 1996; Bireswar Chakrabarti Memorial Lecture in 1999; Dr. D. Govinda Reddy Memorial Award NTR Medical university; Dr. R.V. Rajam Oration award - 2001-2002 by NAMS; Lifetime Service Award in the field of Medical Microbiology by the Rotary Club of Chennai Mid City; Dr. H.I. Jhala award with a Gold Medal by IAMM in 2004. He has been elected as the President of IAMM 2006 – 2007. He receives funding from Indian Federal (CSIR, ICMR, DBT and DST) agencies. He has 160 publications to his credit of which 61 International and 99 National.

INTRODUCTION

AIMS & OBJECTIVES

REVIEW OF LITERATURE

MATERIALS & METHODS

RESULTS

DISCUSSION

REFERENCES

PUBLICATIONS
&
PRESENTATIONS

1. INTRODUCTION

An estimated 1.5 million children throughout the world are blind of whom one million live in Asia (WHO, 1992). Recent blindness surveys from developing countries have shown that 10 % – 40% of childhood blindness is due to cataract (Gilbert et al., 1993, Rahi et al., 1995). Cataract could be traumatic or non – traumatic. The causes of non traumatic cataract are many which includes – Hereditary factors, Congenital Rubella syndrome (CRS) and secondary cataract i.e. cataract due to other ophthalmic diseases like uveitis, aniridia, posterior lenticonus (Eckstein et al., 1996). Clinical and sub - clinical viral infections of the mother can lead to the invasion of the fetus. Thus, the identification of the viruses that have the teratogenic potential is often difficult in the human embryo; since many of the sub - clinical infections go unreported. Viral infections known to produce malformations include Rubella virus (RV), Cytomegalovirus (CMV) and Herpes simplex virus (HSV). A number of other viruses are known to be associated with an increased incidence of illness or fetal death (David et al., 1973). Studies by Eckestein et al., 1996: Angara et al., 1987 and Jain et al., 1983 have shown that in developing countries the congenitally acquired rubella is a common cause of cataract.

RV was first described by two German physicians in the mid- eighteenth century, initially known as “Rotheln”, then as “German Measles”. The devastating effects of RV during pregnancy became more evident during a

later pandemic that swept Europe and the United States in 1963 – 64. RV is an RNA virus, classified as a nonarthropod-borne togavirus, the only member of the genus Rubivirus (Westaway et al., 1985). Although the overall structure and strategy of gene expression is similar to that of alphaviruses of the family Togaviridae, no antigenic relationships have been demonstrated between rubella and other togaviruses nor is there significant homology in their major envelope proteins (Frey et al., 1986; Nakhasi et al., 1986). RV can cause either postnatal or congenital infection. Following respiratory transmission of RV, replication of the virus is thought to occur in the nasopharynx and regional lymph nodes. A viremia occurs 5-7 days after exposure with spread of the virus throughout the body. Transplacental infection of the fetus occurs during viremia. Fetal damage can occur through the destruction of the cells as well as mitotic arrest. Congenital infection of the fetus with rubella is termed as CRS, wherein a triad of organs are affected namely – Heart, eyes and Central Nervous System (CNS).

CMV was discovered in 1956 by Margaret G Smith from the salivary gland of an infected dead infant. It belongs to the genus of Herpes virus, family herpesviridae subfamily betaherpesvirinae. It is a double stranded DNA virus that is enveloped having icosahedral symmetry. CMV is the most common cause of congenital infections due to viruses in the world. Both primary and recurrent infection can result in fetal infection. The birth prevalence of congenital cytomegalovirus infection varies from 0.3% to

2.4%, and at least 90% of congenitally infected infants have no clinical signs (Neto et al., 2004). The major manifestations of cytomegalic inclusion disease (CID) include microcephaly or hydrocephaly, chorioretinitis, blindness, hepatospleenomegaly, encephalitis with cerebral calcifications, and mental retardation (Neto et al., 2004). In contrast to the neurologic damage, extraneural involvement of the liver, spleen, lungs, and kidney is usually reversible with relatively little chance of permanent malfunction.

Herpes simplex virus (HSV) belongs to the genus of Herpes virus, family Herpesviridae and sub family Alphaherpesvirinae. It is a double stranded DNA virus, enveloped having icosahedral symmetry. The recognized serotypes of HSV are HSV serotype 1 and HSV serotype 2. *In utero* infection is rare and only about 0.1 - 0.5 newborns are only infected per 1000 live births. Neonatal infections with HSV –2 are associated with increased mortality. New borns acquire the infections from the genital tract of the mother and the virus may also ascend through the ruptured membranes. It may cause either generalized infections or asymptomatic infections.

The conventional methods of laboratory diagnosis of viral infection include the isolation of virus in cell culture and serological diagnosis. RV grows in a wide range of primary and continuous cell cultures like Vervet monkey kidney (VMK), and Rabbit kidney (RK-13) respectively (Herrmann et al., 1979). Cytopathic effects (CPE) are apparent in the

continuous cell lines like RK- 13, SIRC and some sublines of Vero, if the conditions are properly controlled. Vero cells do not produce interferon and RV can therefore replicate more rapidly to a high titer in these cells (Best et al., 1989). CMV replicates only in human diploid cell cultures and in fibroblast cell cultures producing a CPE. HSV grows in a variety of cell cultures like Veneral Enteric Research Organization (Vero), Statens Serum Institute Rabbit Corneal epithelial cell line (SIRC), HeLa producing the characteristic CPE as Multinucleated Syncyticum formation. These viruses can be isolated from a variety of clinical specimens like respiratory secretions, blood, urine and stools from cases of postnatally acquired rubella and from infants with congenital rubella. They can also be isolated from cataract material, lens fluid, tears, cerebrospinal fluid, and autopsy material from cases of congenital infection; and amniotic fluid, placenta, and fetal tissues from spontaneous and therapeutic abortions. Various serological techniques are employed for the diagnosis of postnatal and congenitally acquired infection and for determining the immune status. Serological techniques include the Single Radial Hemolysis (SRH), Enzyme Immunoassay (EIA), Latex agglutination (LA), Passive Hemagglutination (PHA) and Indirect Immunofluorescence staining (IIF). Virus isolation has the disadvantage of requiring at least 2 weeks to obtain a result. A more rapid method is necessary for it to be useful for the aetiological diagnosis of congenital infections in pregnancy and prenatal diagnosis of congenital infection.

Acute hemorrhagic conjunctivitis (AHC) are usually caused by enterovirus 70 (EV-70), coxsackie A24 (CA 24) and epidemic types of adenoviruses. Epidemics of AHC have occurred in Asia, Africa, and Europe (Langford et al., 1980; Wolken et al., 1974; Kono et al., 1975) and have been recurrent in countries of Asia (Lim et al., 1977). In India, epidemics of AHC occur almost regularly at two to four year intervals, mostly caused by EV-70 and less frequently by CA 24 (Kishore et al., 2001). The infection is primarily localized in the epithelial layers of the conjunctiva and cornea (Mitsui et al., 1972). AHC is characterized by photophobia, watering, foreign body sensation, eyelid edema, conjunctival hemorrhages and superficial punctate keratitis (Maitreyi et al., 1999).

Human enteroviruses are RNA viruses in the family Picornaviridae and are classified into Polioviruses, Coxsackie A viruses, Coxsackie B viruses and ECHO viruses. Enteroviruses are assigned with specific consecutive numbers based on the pathogenesis in humans and experimental animals. There are 66 immunologically distinct serotypes (Muir et al., 1998). Coxsackie viruses are typical picornaviruses. By neutralization test (NT) Group A viruses are classified into 24 types and group B viruses into six types. The conventional methods used for the isolation and identification of the enteroviruses are cell cultures and neutralization with pooled virus specific antisera. These methods are however labor – intensive and time consuming and require large pools of reference antisera. Hence these tests have a limitation in that they can be performed only in specified reference

laboratories. Moreover, new strains of viruses are untypeable, and negative cell culture results for clinical specimens are non - diagnostic (Muir et al., 1998; Oberste et al., 2000). These disadvantages in the conventional methods necessaites the need for the more rapid and specific nucleic acid based amplification methods.

Adenoviruses (Ad) are DNA viruses belonging to the family Adenoviridae, which consists of two genera: Mastadenovirus, the adenovirus of the mammals and Aviadenovirus, the adenovirus of the birds. All mammalian adenoviruses share a common complement-fixing antigen (CFA). Serotypes are distinguished by NT. Human adenoviruses is composed of 51 serotypes that have been grouped into six different species, A to F based on their physiochemical, biological and genetic properties (Jong et al., 1999; Wandell et al., 1984). Medically important species in subgenus D are adenovirus types 8, 19 and 37. They mainly cause epidemic keratoconjunctivitis (EKC) (Noda et al., 1991). Adenovirus infections of the eyes can present as epidemic keratoconjunctivitis, pharyngoconjunctival fever or follicular conjunctivitis. Usually Keratoconjunctivitis in patients with conjunctival inflammation disappears with 2- 4 weeks, whereas keratitis may persist longer. Patients with EKC are infectious during the first 2 – 3 weeks of infection and transmission occurs via fomites, which include contaminated towels or contaminated articles of daily use in schools, clinics and swimming pools. To prevent transmission and outbreaks with the hospital,

appropriate disinfection of hands and ophthalmological instruments must be done. No specific treatment is available (Schrauder et al., 2006). Adenoviruses are host specific and they grow only in cell cultures of human origin, such as human embryonic kidney, HeLa or HEp – 2. The typical CPE consists of rounding and aggregation of the cells into grape like clusters. Intranuclear inclusions may be seen in stained preparations (Ananthanarayanan et al., 1996). The laboratory diagnosis of adenovirus infection is done usually by inoculating the relevant clinical specimen into tissue cultures. Preliminary identification is possible by the CPE production and confirmed by Complement Fixation test (CFT) with adenovirus antiserum. Hemagglutination with rat and monkey erythrocytes, the isolate can be further classified into subgroups. Serotyping of the isolates is done by the neutralization test. Serological diagnosis is usually done by demonstrating a rise in titre of antibodies in the paired serum sample. Examination of a single sample of serum is inconclusive as adenovirus antibodies are common in the population.

Almost all the viruses that cause any clinical infection can be cultured in appropriate cell lines, this conventional technique requires an established tissue culture laboratory and the results are usually available only after 2 – 3 weeks. Moreover, maintenance of tissue culture laboratory is very expensive and requires expertise. Hence, the trend is towards more rapid molecular biological diagnostic techniques based on nucleic acid methodologies, which increasingly are replacing viral culture methods. In

this study, standardization and application of nucleic acid based molecular biological techniques for rapid detection, quantification and characterization of specific viral etiologic agents for the identification of serotypes causing infections have been carried out.

2. Objectives

2. 1 Hypothesis:

Nature of genotypes and serotypes of viruses causing congenital cataract and epidemic conjunctivitis in Indian patients may be different from those reported from other parts of the world.

2. 2 Overall aim:

Standardize and apply nucleic acid based molecular biological methods for the detection and characterization of viral etiological agents associated with the causation of congenital cataract and epidemic conjunctivitis in the hospital-based patients.

2.3 AIM:

2.3.1 Standardization of nRT-PCR for the detection of RV in clinical specimens.

a. Comparison of three RNA extraction methods viz., QIAGEN kit method, TRIZol method, conventional method of GTC.

b. Standardize a RT-PCR using nested set of primers targeting the E1 gene of RV within the molecular epidemiological window for the detection of RV in clinical specimens.

c. Application of the standardized nRT-PCR onto various clinical specimens for the detection of RV.

2.3.2 To standardize the conventional method of virus isolation for RV.

a. Conventional method of virus isolation using cell cultures.

b. Standardize IIF staining for the detection of RV from cell cultures.

c. Apply standardized nRT-PCR for the confirmation of RV isolation using cell cultures.

2.3.3 To characterize the RV isolates by genotyping.

a. Standardization of DNA sequencing technique for the rapid sequence determination of RV isolates.

b. To apply the MultAlin software for genotype determination of RV isolates.

2.3.4 To determine the viral etiology in congenital cataract by nucleic acid based amplification techniques.

a. To apply the standardized snPCR for the detection of HSV in Lens aspirates (LA).

b. Application of nRT-PCR onto LA for the detection of RV.

c. To apply the standardized nPCR for the detection of CMV in LA.
d. Application of the optimized snPCR for the detection of VZV in LA.
e. To standardize the Real Time PCR technique for the quantitation of HSV viral particles detected in LA.
f. To determine the RV infection using serological diagnosis and to compare it with molecular methods.

2.3.5 Standardization of snPCR for the rapid detection and identification of HSV serotypes in culture negative intraocular aspirates.

a. Standardize a snPCR using semi nested set of primers targeting the Glycoprotein D gene of HSV for the rapid detection and serotype identification of HSV in clinical specimens.
b. Application of the standardized snPCR onto culture negative intraocular aspirates for the detection of HSV.
c. Standarization of DNA sequencing technique to serotype HSV and to compare the results with snPCR technique.
d. Application of uPCR targeting the DNA Polymerase gene of HSV for the detection of HSV in clinical specimens and to compare the results with snPCR targeting the Glycoprotein D gene.

2.3.6 Application of snPCR for the detection of HSV from various clinical specimens.

a. To standardize and apply PCR based Restriction Fragment length Polymorphism (PCR – RFLP) for the identification of HSV serotype.

2.3.7 To determine the causative viral agents of epidemic acute hemorrhagic conjunctivitis.

a. To apply a standardized nPCR for the detection of Ad from conjunctival specimens collected in the epidemic.
b. To standardize a uRT-PCR for the detection of CA 24 variant from conjunctival specimens collected during the epidemic.
c. To attempt isolation of viruses associated with conjunctivitis using cell cultures.
d. To standardize the DNA sequencing technique for the rapid serotype identification of Ad isolates.
e. To attempt isolation of CA 24 variant using cell cultures.
f. To determine sequence variations if there are any and to characterize the uPCR positive CA 24 specimens by DNA sequencing.
g. To detect and identify other enteroviruses causing conjunctivitis using panenterovirus primers in clinical specimens negative for Ad and CA 24 variant.

3. REVIEW OF LITERATURE

3.1 CONGENITAL VIRAL INFECTIONS:

Infectious agents have long been recognized as a cause for spontaneous abortion or perinatal mortality. A typical pregnancy lasts for a period of 40 weeks with the first trimester lasting for 12 weeks, second trimester starting from 13 – 27 weeks and the last trimester from 28th up to 40th week. Prenatal infections are defined as those infections acquired just preceding the birth. Perinatal infections are defined as those infections acquired from 20-29th week of pregnancy up to 28th day after birth. Thus it is important for a pregnant woman to know the basic mechanism of infections that can potentially affect pregnancy outcome, so that it can be prevented (Chernesky et al., 1984).

The mechanisms involved can be broadly classified into three as-

1. Ascending infections
2. Transplacental infections
3. Infections acquired through the birth canal.

3.1.1 Ascending infections:

Ascending infections occur when the microorganisms residing in the external genitalia of a pregnant woman gain access to the amniotic sac. This can eventually lead to the rupturing of the sac, thus facilitating the spread of the infectious agent into the amniotic fluid. The fetus inside the

womb gets infected by aspirating the microorganisms into the lungs, by swallowing them or by penetration of the organisms through the ear canal. These events in turn lead to an inflammatory response on the amniotic sac triggering labor (Evaldson et al., 1982).

3.1.2 Transplacental infections:

In cases of transplacental infections, the mother has an infection along with the presence of circulating microorganisms in the blood. Due to the presence of the microorganism in the blood of the mother, the organisms infect the fetus through the placenta (Koi et al., 2001).

3.1.3 Infections acquired through the birth canal:

Some microorganisms cannot ascend the amniotic sac or cross the placental barrier, in such cases they colonize the external genitalia of the pregnant women.

However during delivery the fetus is contaminated / infected by the exposure to maternal blood and other secretions at the birth canal. Viral infections in pregnancy are major causes of mortality and morbidity for both the mother and the fetus. Infections can occur in the neonate transplacentally, perinatally or post natally (Koi et al., 2001). The viral infections of major concern during pregnancy are the following- Rubella virus, Cytomegalovirus, Herpes simplex virus, Varicella zoster virus, Human Parvo virus B19, Measles virus, Human immunodeficiency

viruses, Hepatitis B virus. World wide congenital HIV infection is now a major cause of infant and childhood morbidity and mortality, with an estimated 4 million deaths occurring since the start of the pandemic (Helene et al., 2001).

3.2 RUBELLA VIRUS:

This virus was first discovered by two German physicians de Bergan and Orlow in 1870s. The association of the virus with the maternal infection and congenital defects were established only in early 1940s by N McAlister Gregg. It belongs to the family of Togaviridae, genus Rubivirus. It is a single stranded enveloped RNA virus having icosahedral symmetry. Mother contracts the infection from the infected respiratory droplets, with development of viremia and the chorionic layers of the placenta become infected leading to necrotic changes and the cells get desquamated into the lumen of vessels and thus enter the fetal circulation leading to disturbance in organogenesis (Webster et al., 1998). The rate of malformations and fetal abnormalities depends on the gestation period of maternal infection. In general, the earlier in pregnancy the mother contracts rubella, the greater the risk of severe generalized involvement of the fetus. Infection during the first six weeks, the frequency of rubella embryopathy is highest and as the weeks pass by, the chorionic layers becomes impermeable to RV infection and after 22 weeks of gestation period, the rate of rubella embryopathy is nil. The teratogenicity is highest

in the first trimester, less in the second and none in the third (Best et al., 1989). Since then, the fetal defects have been characterized as the Congenital Rubella Syndrome (CRS) with defects that can be classified into transient, developmental and permanent types (Tang et al., 2003). Fetal damage is multifactorial; resulting from combination of rubella virus- induced cellular damage and the effect of the virus on dividing cells. The clinical manifestations of congenital rubella are listed (Best et al., 1989) -

Eyes: Retinopathy, Cataract and Microphthalmos

Ears: Uni or bi lateral deafness and Deafness associated speech defects

Heart: Patent ductus arteriosus, Pulmonary arterial stenosis and Ventricular septal defect

Central nervous system: Mental retardation, Central auditory defects, Sensoneural defects

General: Low birth weight, Micrognathia and Fetal loss (Rare)

Transient neonatal effects: Adenopathies, Bony radiolucencies, Hematospenomegaly, Meningoencephalitis Thrombocytopenia with or without purpura

Late emerging / Developmental: Chronic diarrhea, Late onset of intestinial pneumonitis and Insulin dependent diabetes mellitus

3.2.1 Placental infection:

During the period of maternal viremia the placenta may become infected. Although the virus may persist for months in the placenta, recovery of the virus from the placenta at birth is infrequent. In a study conducted by Tondury *et al*, where they examined the chorion obtained from 12 specimens 10 to 45 days after the occurrence of maternal rubella, eight of the specimens showed necrotic foci in the epithelium of the chorionic villi and in the endothelium of the capillaries and lager vessels. Damaged endothelial cells were observed to be desquamated into the lumen of the vessels and they enter the fetal circulation by embolic transport. Infection at later stages of pregnancy causes multifocal chronic mononuclear cell infiltrates in the placental membranes, cord and deciduas, along with vasculitis; these culminate in placental hypoplasia and placentitis (Atreya et al., 2004).

3.2.2 Heart defects:

The virus enters the early embryo and induces a chronic non- lytic infection. Presumably, spread of the virus is initially through the vascular system, and many of the first trimester abortuses show non-inflammatory damage to the endothelial cells lining blood vessels. Direct viral damage of the septa of the heart may be the cause of increased incidence of the septal defects associated with first trimester infection. Damage to

endothelial cells can also lead to thrombosis of small vessels and surrounding tissue necrosis. The absence of any inflammatory reaction in the infected fetal tissues in the first trimester is characteristic. The most common cardiovascular lesions are patent ductus arteriosus associated with infection 11 to 48 days after fertilization and stenosis of the pulmonary artery and its branches 16 to 57 days after fertilization (Webster et al., 1998).

3.2.3 Patent Ductus Arteriosus:

The ductus arteriosus is a blood vessel that connects the pulmonary artery and the aorta in the fetus allowing the blood to bypass circulation to the lungs. Since the fetus does not use his / her lungs as oxygen is provided through the mother's placenta, flow from the right ventricle needs an outlet. The ductus provides this, shunting the flow from the left pulmonary artery to the aorta just beyond the origin of the artery to the left subclavian artery. The high levels of oxygen, which is exposed after birth causes it to close in most cases within 24 hours. When it doesn't close, it is termed as "Patent ductus arteriosus". The defect corrects by itself within several months of birth, but may require infusion of chemicals, the placement of "plugs" via catheters, or surgical closure. The flow pattern is similar to the septal defects noted above, except that the shunting occurs outside of the heart. The left ventricle has to pump blood out through the aorta, only to have some of it flow to the lower pressure pulmonary artery, and directly

back to the left atrium and ventricle. If a large PDA is not corrected, then the pressure in the pulmonary arteries may become very high and induce changes in the arteries themselves such that even closure of the defect will no longer improve the patient. In this case, the pressure in the right side of the heart is high enough that blood may begin to flow from the right to the left side of the heart. This situation is called "Eisenmenger's syndrome" (Pflieger et al., 2003).

3.2.4 Pulmonary Artery Stenosis:

Pulmonary artery stenosis is a narrowing that occurs in pulmonary artery, a large artery that sends oxygen – poor blood into the lungs for oxygenation. The narrowing may occur in Pulmonary artery and or in the left or right pulmonary artery branches. The narrowing makes it difficult for blood to reach the lungs to pick up oxygen. Without enough oxygen, the heart and body cannot function, as they should. In an effort to overcome the narrowing, the pressure in the right ventricle rises to levels that can be damaging to the heart muscle (Pflieger et al., 2003).

3.2.5 Eye defects:

Opacities in the primary lens fibres resulting in a characteristic central or nuclear cataract were the unique symptoms observed by Gregg et al in 1941. Involvement of the central lens fibres indicates that the cataractous process has begun early in the life of embryo. Lenses from the first

trimester rubella infected abortuses showed pyknotic nuclei, cytoplasmic vacuoles, and inclusion bodies in the primary lens cells and retardation of lens development. Late changes included degeneration of some primary lens fibres and evidence of active disease in the newly developing equatorial lens fibre cells, indicating chronic infection. Retention of the nuclei in surviving lens fibres appears to be characteristic of rubella cataracts. In some CRS cases there is damage to other ocular structures including focal necrosis of the pigment epithelium of the retina and necrosis of the ciliary body and iris as well as Microphthalmia. Retinopathy and glaucoma occur after infection over a much longer period (2-117 days gestation) than do cataracts. The rubella virus is cytopathic to some of the lens fibres in the developing eyes causing degeneration of the primary lens fibres and damaging the newly formed lens fibres, resulting in a whitish haze towards the lateral border of the lens (Webster et al., 1998).

3.2.6 IRIS & CILIARY BODY INFECTION:

If the virus infects the fetus at the 7th -8th week of gestation then the development of the iris, ciliary body and anterior chamber angle may be disturbed. The pigment epithelium of iris and ciliary body can necrotize and vacuoloze. The iris dilator muscle and the stroma can become atrophic. Since the fetal immune apparatus has not yet developed they fail to mount an immune response but contrastingly chronic anterior uveitis

may be seen postnatally. This persistent nongranulomatous inflammation can cause permanent damage to the anterior angle structures (Lee et al.1999).

3.2.7 RETINOPATHY:

In his original paper Gregg had mentioned an observation made by Dr. Aileen Mitchell in a case of monocular cataract. The fundus showed pale and some scattered irregular-shaped spots of pigment, subsequently “RUBELLA RETINITIS” came to be recognized as the most frequent abnormalities in congenital rubella, with a frequency rate of 13% to 61%. It is typically a stationery abnormality characterized by the widespread pigmentary disturbance, most marked in the macular area but fortunately not accompanied by the functional alteration in the sensory retina. Tremendous variations are seen in the description of this retinopathy as bone-spicule like, mottled, finely stippled or blotchy. The condition can be either bilateral / unilateral which can be seen in the central area or in the periphery. Rubella retinopathy can be progressive with the uneven development of retinal pigment epithelium. It may also lead to Sub- retinal Neovascularization (Lorenz et al., 1968; Orth et al., 1980).

3.2.8 CATARACT:

Maternal rubella infection in the first trimester may result in cataract formation in the fetus. Intraocular virus that is present can dramatically

slow the fetal cell replication rate. The decrease in the rate of replication results in the retention of the nuclei in the lens fibre in the normally acellular nuclear portion of the lens. Gregg in his original paper has described the cataract as follows- "In the undialated condition of the pupil the opacities fill the entire area. After dilatation the opacities appeared densly white – sometimes quite pearly in the central area, with a small, apparently clear, zone between this and the papillary border of the iris. The cataractous process seemed to have occurred in all but the outermost layers of the lens, and was considered to have begun early in the life of the embryo." Hence the cataract in rubella-infected patients is always dense centrally with less opaque peripheral cortex and capsule. Less often there is thinning of the lens. Congenital aphakia secondary to cataract has also been described. The virus persists in the lens for at least a year in majority of the patients (Lorenz et al., 1968).

3.2.9 MICROPHTHALMOS:

The relationship of mild microphthalmos to congenital cataract has been observed frequently. If the cataract is present in only one eye, that eye tends to be smaller than the other eye. The unilateral cataract formation reflects the inability of the virus to invade or establish itself in the noncataractous lens, but it is well established that the retinopathy of the congenital rubella is often visible in the noncataractous eye. Associated with microphthalmos is the presence of iridocyclitis and focal necrosis of

the pigment epithelium of the ciliary body, which is seen, in the clinically normal eye of the cases of congenital rubella. The greater the degree of parasitism of cells of the embryonic eye, the greater are the chances that the lens will be affected and greater will be the effect of the virus on overall growth of the eye. Thus it has been proposed that the condition microphthalmos may simply reflect the general tendency for the virus to slow down growth of the cells (Lorenz et al., 1968).

3.2.10 GLAUCOMA:

Approximately 10 % of children with congenital rubella will develop glaucoma. The causes include chronic inflammation, enlarged cataractous lenses causing angle closure. Glaucoma may not be recognized until after the perinatal period (Lorenz et al., 1968).

3.2.11 Ear defects:

Sensorineural deafness, which can progress after birth, is the most common rubella associated defect and mainly results when rubella infection occurs in the first 16 weeks of gestation. It is likely that the virus gains access to the inner ear through the blood supply of the stria vascularis, leading to the damage of the epithelium of the cochlear duct. On going viral infection and or immunological mechanisms, such as immune complex deposition in small vessels, may lead to further damage (Webster et al., 1998).

3.2.12 Brain defects:

Brain damage only occurs after rubella infection in the first 16 weeks of gestation, causing mild to severe mental retardation with spastic diplegia, ischemic damage and variable Microcephaly. Ongoing viral infection of the endothelial cells and or subsequent damage by immune complexes may lead to progressive vascular damage in the prenatal and postnatal brain. Microcephaly in CRS infants would be consistent with the concept that the virus can infect the neuroepithelium and reduce cell proliferation (Webster et al., 1998). The infected infants exhibit the following features- Retinopathy, cataract, patent ductus arteriosus, pulmonary artery stenosis, deafness, thrombocytopenia, blueberry muffin skin lesions. Thus pregnant women who are exposed to rubella should have serological tests done for the evaluation of IgG and IgM antibodies to rubella virus. If primary rubella is diagnosed in the first trimester of the pregnancy, the mother should be counseled about the option to terminate the pregnancy (Robert et al., 2004).

3.3 Diagnosis of congenitally acquired infection:

The diagnosis of congenitally acquired rubella is made by –

1. The presence of Rubella IgM in cord blood or serum samples taken in infancy.
2. Detection of rubella antibodies at a time when maternal antibodies should have disappeared (approximately at 6months of age).

3. Isolation of rubella virus form infected infants in the first few months of life.

   The detection of rubella specific IgM in cord blood or infant sera is the method of choice for the diagnosis of congenital rubella. Specific IgM has been demonstrated in all confirmed cases to the age of 3 months, in 86% 3 to 6 months, 62% 6 months to 1 year and 42% 12 to 18 months and rarely over 18 months. If the IgM result is negative or equivocal and if there is going to be a history of rubella infection during pregnancy, a serum sample can be collected at the end of 9-12 months to look for the presence of specific IgG. Since rubella is uncommon under the age of 2 years, IgG detected between 1 and 2 may be indicative of congenital infection. However each case must be assessed individually taking into account factors such as age, maternal history, and presence of clinical findings (Best et al., 1989).

3.4 Prenatal diagnosis of congenital rubella infection:

Prenatal diagnosis of congenital infection may be of value when maternal infection occurs after the first trimester, in cases of maternal reinfection and in cases where equivocal serology results from the mother were obtained. Possible methods include-

1. The testing of fetal blood sample obtained by fetoscopy for rubella specific IgM. However the fetus does not produce sufficient IgM for detection before 22 weeks.

2. Virus may be isolated from amniotic fluid but the reliability of this technique has not been demonstrated.
3. The detection of rubella RNA or viral proteins in chorionic villus biopsies and amniotic is currently being evaluated (Best et al., 1989).

3.5 Prevention:

Vaccines:

A number of rubella vaccines have been developed. RA27/3 is now the most commonly used vaccine strain throughout the world. All vaccines are administered subcutaneously. Virus is excreted for 1- 4 weeks after vaccination, but is not transmitted to susceptible contacts (Halstead et al., 1971; Scott et al., 1971). Rubella vaccines are well tolerated although rash, lymphadenopathy, and arthropathy may occur between 10 days and 4 weeks after vaccination (Best et al., 1974; Perkins et al., 1985). Licensed Rubella vaccines are many which have been tabulated in table 1.

3.6 Vaccination during pregnancy:

Rubella infection during pregnancy is contraindicated since it may result in fetal infection, although evidence of fetal damage are not reported (Preblud et al., 1981).

Table 1: Licensed Attenuated Rubella vaccines

Vaccine	Attenuation
HPV77.DE5	VMK, Duck embryo fibroblasts
Cendehill	VMK, Primary rabbit kidney
RA27/3	Human Embryonic kidney, WI-38 fibroblasts
Takahashi (KRT vaccine)	VMK, primary rabbit testicle cells, rabbit kidney
Matsuura (QEF, MEQ11)	VMK, Chick amnion, Japanese quail embryo fibroblasts
To-336	**VMK, primary guinea- pig kidney, primary rabbit kidney**
Matsuba (SK vaccine)	VMK, swine kidney, Primary rabbit kidney
TCRB19	VMK, bovine kidney, rabbit kidney
BRD-2	Diploid human cells

3.7 Cytomegalovirus:

It was discovered in 1956 by Margaret G Smith from the salivary gland of an infected dead infant. It belongs to the genus of Herpes virus, family herpesviridae subfamily betaherpesvirinae. It is a double stranded DNA virus that is enveloped having icosahedral symmetry. Cytomegalovirus is the leading cause of congenital infection in the developed countries, occurring in 0.3 - 2% all live births. The placental development takes place in a sequential manner in which the epithelial stem cells differentiate into cytotrophoblasts which forms either the anchoring / floating villi. If they aggregate into columns of single cells they form the anchoring villi and if the cells fuse they form floating villli and thus the cells are called as syncytiotrophoblast. The anchoring villi get attached to the uterine wall and the cytotrophoblasts invade the uterine wall which helps in the anchorage of the fetus to the mother and hence in turn come in contact with the maternal circulation. The infected maternal leucocytes come in contact with the cytotrophoblasts of the anchoring villi and the infected cytotrophoblasts transmits it to the villous cores and the fetal endothelial cells are in turn infected. Mother gets the infection primarily from infected salivary / other secretions /through sexual contact and transmits it to the fetus via the placenta. The important fact that is to be remembered about CMV is that it can infect the fetus at

any stage of the pregnancy. *In utero* transmission of the CMV can lead to either primary infection /recurrent latent viral reactivation. The virus can cause either symptomatic / asymptomatic infection. In congenital CMV infections, over 90% of the new borns are asymptomatic at the time of birth but the consequences of *in* utero CMV infection such as hearing loss will show up as the infants gets older. Infection in the infants if symptomatic causes cytomegalic inclusion disease. If asymptomatic it later results in extensive hearing deficit and impaired development (Maria et al., 2002). The manifestations of cytomegalic inclusion disease in infants are the following- jaundice, microcephaly, chorioretinitis, cerebral calcifications, seizures, mental retardation and premature birth. In contrast to the neurologic damage, extraneural involvement of the liver, spleen, lungs, and kidney is usually reversible with relatively little chance of permanent malfunction. Babies may also have impaired cell - mediated immunity. The most morphological abnormalities is Microcephaly and periventricular calcification. Death that occurs shortly after birth tends to be resulted from hepatic failure. CMV infections due to primary infections produce more severe damage than those resulted from reactivation of latent infection. Hence when primary CMV infection or maternal CMV viremia is diagnosed during pregnancy, prenatal diagnosis should be done. Although transmission of CMV from mother to fetus may occur at any time during pregnancy, severe neurological

complications have been reported to occur when a primary infection or high viral load infection is diagnosed during the first half of gestation (David et al., 1973; Landini et al., 1999).

3.8 Herpes simplex virus:

It belongs to the genus of Herpes virus, family Herpesviridae and sub family Alphaherpesvirinae. It is a double stranded DNA virus, enveloped having icosahedral symmetry. *In utero* infection is rare and only about 0.1-0.5 newborns per 1000 live births. Neonatal infections with HSV 2 are associated with increased mortality. Newborn infection with HSV type 1 or type 2 can have devastating consequences (Enright et al., 2002; Whitley et al., 2002; Kohl et al., 1997). Presentations include disease limited to the skin, eye or mucous membranes (SEM disease), the central nervous disease (CNS disease) or disseminated disease. Even with therapy, the risk of long term morbidity, particulary neurodevelopmental morbidity, may be significant (Schleiss et al., 2003). The seroprevalence of HSV infections, including genital herpes, in the developed world is increasing, leading to an apparent increase in the incidence of neonatal herpes (Fleming et al., 1997). The most severe infections will result in a triad of symptoms-

1. Skin vesicles or scarring

2. Eye disease - chorioretinitis along with other eye findings like keratoconjunctivitis
3. Microcepahly or Hydranencephaly

   In persons with asymptomatic infection localization of the virus occurs in central nervous system, skin, mouth, eye, etc and the mortality rate is lower than 20%. If it is known that the mother has active herpes during labor, prolonged rupture of membranes and fetal scalp electrode monitoring should be avoided. The baby should be delivered only by elective caesarean section because of the risks of infection with a vaginal delivery. However caesarean section does not always prevent perinatal infection (John et al., 2000).

3.9 Varicella zoster virus:

It is placed under the genus Herpes virus, family Herpesviridae and sub family Alphaherpesvirinae. It is a double stranded DNA virus enveloped having icosahedral symmetry. The attack rate among susceptible contacts is 60 - 90% after exposure. The incubation period after infection is 10 - 20 days, with a mean of 14 days. In pregnancy, varicella may be transmitted across the placenta, resulting in congenital or neonatal chickenpox (Robert et al., 2004). The risk of congenital varicella syndrome is limited to exposure during the first 20 weeks of gestation, occurs uncommonly and is characterized by skin scarring, limb hypoplasia, chorioretinitis, and Microcephaly. Neonatal

VZV infection is associated with a high neonatal death rate when maternal disease develops from five days before delivery up to 48 hours post partum as a result of the relative immaturity of the neonatal immune system and the lack of protective maternal antibody. By lab testing if the mother is found to be susceptible then the mother should be treated with Varicella zoster Immunoglobulin intramuscularly within 96 hours to prevent the maternal infection, but in case she develops infection should be treated with Acyclovir (Schleiss et al., 2003).

3. 10 LABORATORY DIAGNOSIS OF CONGENITALVIRAL INFECTIONS:

The basic approaches to the laboratory diagnosis of viral infections are the same as they have been for decades, viz., direct demonstration of virus or viral products in clinical specimens, isolation and identification of virus in a susceptible host system, and antibody assays for serodiagnosis or determination of immune status. However advances have been made in each of these areas, which permit results to be obtained more rapidly and efficiently. A variety of molecular methods are also now available for analysis of viral nucleic acids. These permit more precise differentiation of viral strains that can be achieved by antigenic analysis, and they are very useful in epidemiological studies (Schmidt et al 1979).

Many rash illnesses may mimic rubella infection and upto 50% of rubella infections may be subclinical. The only reliable evidence of acute rubella infection is the presence of rubella – specific IgM antibody, demonstration of a significant rise in IgG antibody from paired acute and convalescent serum samples, or a positive viral culture for rubella, or detection of Rubella virus by molecular methods like Reverse transcriptase polymerase chain reaction (RT-PCR) (Best JM et al., 1989).

Rubella isolation is extremely labor intensive and time consuming. Rubella virus RNA has been detected in the fetal specimens by nucleic acid hybridization, but false negative results have been reported. A potentially more sensitive technique to detect rubella virus RNA is reverse transcription followed by PCR amplification (Bosma et al., 1995).

3.10.1 DIRECT DETECTION OF VIRUS IN CLINICAL SPECIMENS:

Methods which permit the direct demonstration of virus or viral products in the clinical specimens avoid the need to cultivate the virus in a living host system, and thus are more rapid and economical. Further they permit the detection of viruses which cannot be cultivated by standard procedures, or which are no longer infectious in the specimen. Methods currently available for direct virus detection in clinical specimens include electron microscopy (EM); various

immunoassays which employ antibody probes, such as immune electron microscopy (IEM), immunofluorescence (IF) – fluorescent antibody staining (FA) and immunoperoxidase (IP) staining, enzyme immunoassay (EIA), radioimmunoassay (RIA) and latex agglutination (LA) tests; and nucleic acid hybridization procedures for detection of viral genomic material.

The FA staining is the most widely used test in the diagnostic virology. Rapid viral diagnosis by means of FA staining was first described by Liu *et al* in 1956 for detection of influenza and was pioneered for numerous viruses by Gardener and McQuillan et al in 1980. This method was widely adopted by the clinical labs during the 1980s, particularly for the detection of respiratory viruses. FA staining techniques continue to be improved through the use of cytocentrifugation of specimens and simultaneous staining with different antibodies labelled with different fluorescent labels (Landry et al., 1997; Landry et al., 2000 a, Landry et al 2000 b). The viral antigen detection can be done by immunofluorescence in a huge variety of specimens which includes- staining the nasopharygneal aspirates for respiratory viruses, Feces for Rotavirus, Blood specimen for pp65 antigenemia for Cytomegalovirus, Skin scrapings in case of detection of herpes viruses like- Herpes simplex virus, Varicella zoster virus, conjuncitval swabs for the detection of adenoviral / enterovirus in severe epidemic conjunctivitis situations. Immunofluorescence

staining can be either direct or indirect staining. Direct staining technique is the one in which the antigen which is expressed on the surface of the virus attaches to the labelled antibody that is being added and the antigen is detected by the labelled antibody. In case of the in – direct method the antigen binds with the unlabelled antibody and then an anti- antibody tagged with fluorescence is added and it is visualized. The main advantage of using this immunofluorescence staining is that there is timely reporting of either the presence / absence of the viral antigen based on which the clinician decides about the antiviral treatment. The main disadvantages associated with this technique are that the sensitivity of this technique is not so good and there are all the more chances that we may miss the presence of the viral antigen. The results also vary between individuals and trained staff personnel are required to report the results. The results of this have to be verified by isolating the virus described (Landry et al., 2000a).

3. 10. 2 ELECTRON MICROSCOPY:

The application of electron microscopy to the rapid diagnosis of viral infections is an important and integral part of the battery of diagnostic procedures currently available. It often serves as an adjunct to other diagnostic procedures, but it in a few instances it is the primary method of viral diagnosis (Schmidt et al., 1989). Electron microscopy

will permit the detection of wide range of infectious agents from a variety of clinical specimens. Electron microscopy can be either direct or immunoelectron microscopy. With direct methods, negative staining is normally used, which require very little special equipments, in contrast to thin sectioning techniques. The specimens can be used either directly or the viral particles can be concentrated before negative staining. Several methods are available for concentration, including differential centrifugation, ammonium persulphate precipitation and agar diffusion method. Immunoelectron microscopy is by means of increasing the sensitivity and specificity of EM and is particularly useful in the following situation- especially when the number of virus particles present is small. The IEM can be further classified into simple IEM and solid phase IEM. In the simple IEM the specimen is incubated with the specific antibody before staining in the hope that the antibody will agglutinate the specimen and then Negative staining is done. In case of the solid phase IEM the copy grid is coated with specific antibody, which is used to capture virus particles from the specimen.

The main advantage of this technique is that there is visualization of the virus that helps in the rapid diagnosis. The disadvantages are many to list out few of which are that multiple specimens cannot be examined coincidentally and the minimum number of viral particles

needed for the visualization is 10^{6} / ml of the specimen (Schmidt et al., 1989).

3. 10. 3 LIGHT MICROSCOPY:

The light microscopy is used to visualize the histologic changes in the virus infected cells. The changes can be either in the cytoplasm or the nucleus. The changes seen are specific for viruses. The specimens to be collected include the scrapings from the base of the lesions, and in case of fluids a cytospinned smear is preferred. The cellular material can also be concentrated by membrane filtration technique. The cells concentrated can be made into a cellblock and sections can be taken and stained accordingly to see the changes. In case of a HSV infection the changes seen are that the nucleus becomes ground glass in appearance and multinucleated intranuclear inclusions are seen. In case of a CMV infection multinucleated giant cells are seen. The disadvantages are that low sensitivity and specificity and nonspecific changes can be mistaken for infection (Landry et al.,2000 c).

3. 10.4 ISOLATION OF VIRUSES:

The modern era of diagnostic virology dates to the first description of the viral isolations in cell culture by Weller & Enders et al in 1948 and by Enders et al in 1949. Indeed, the need for cell culture technique is the reason for virology laboratories a entities separate form other

general clinical microbiology laboratory. Viruses are obligate intracellular parasites and hence it needs a living cell to infect and sustain its infectivity. In laboratory the viruses can be cultivated by any of the three methods - inoculating into susceptible lab animals, embryonated eggs or inoculation into cell cultures (Ananthanarayan et al., 1984).

3. 10. 5 INOCULATION INTO LAB ANIMALS:

The earliest method for the cultivation of the viruses causing human diseases was by inoculating into human volunteers. Reed *et al* in 1900 used human volunteers for their pioneering work on yellow fever, but due to the serious risk involved the lab animals were used later for cultivating the viruses. Monkeys were first used for the isolation of polioviruses by Landsteiner and Popper in 1909, but due to their cost and risk to the handlers monkeys find only limited application in virology. The use of white mice pioneered by Theiler in 1903 extended the scope of animal inoculation greatly. Mice are still the most widely employed animals in virology. Suckling mouse is found to be a susceptible animal for cultivation of the coxsackie and Arbo group of viruses. The various routes of inoculation of the viruses into animals include intraperitoneal, intracerebral, subcutaneous or intranasal etc. The growth of the viruses inoculated animals may be indicated by either death of the animal, disease or visible lesions produced in the

diseased animal. Disadvantages of animal inoculation are that immunity of the animal may interfere with the viral growth and that animals often harbor latent viruses (Ananthanarayan et al., 1984).

3. 10. 6 INOCULATION INTO EMBRYONATED EGGS:

The embryonated hen's egg was first used for the cultivation of viruses by Goodpasture in 1931 and the method was further developed by Burnett. The embryonated eggs offer several sites for cultivation of the viruses. The isolation depends on the route of inoculation & also on the quality of the eggs. The embryonated eggs have a shell covered by a shell membrane followed by the chorioallantois the respiratory organ, allantoic cavity & the amniotic cavity and then the yolk sac in which the embryo grows. The various route of inoculation of the viruses into the eggs are – amniotic, chorioallantoic, allantoic & yolk sac. Amniotic route is preferred for those viruses that infect the respiratory system like Influenza. Inoculation into the chorioallantoic membrane produces visible lesions/ pocks. Different viruses have different pock morphology. Under optimal conditions each infectious viral particle can form one pock. Hence chorioallantoic is preferred for pock forming viruses like the Variola and vaccinia. Inoculation into the allantoic cavity produces a rich yield of influenza and paramyxoviruses. Yolk sac is preferred for cultivating Chlamydia & Rickettsiae. Eggs should be selected in such a way that only fertile

eggs to be used for inoculation and the eggs should be humidified in the chamber for 4-5 days before inoculation. All the eggs should be candled and verified before inoculation. Duck eggs are bigger and have a longer incubation period than hen's egg. They therefore provide a better yield of Rabies virus and are used for preparation of the inactivated nonneural rabies vaccine (Landry et al., 2000 c, Ananthanarayan et al., 1984).

3. 10.7 CELL CULTURE TECHNIQUE:

Cultivation of bits of tissues and organs *in vitro* have been utilized by physiologists and surgeons for the study of morphogenesis and wound healing. Probably the first application of tissue culture in virology was by Steinhardt *et al* in 1913 who maintained the vaccinia virus in the fragments of rabbit cornea. Maitland in 1928 used chopped tissues in nutrient media for cultivation of vaccinia virus. The major difficulty faced was that of the bacterial contamination which was later overcome by the introduction of the antibiotics (Ananthanarayan et al., 1984). The major turning point, which made tissue culture, the most important method for cultivating the virus was the demonstration by Enders, Weller & Robbins *et al* in early 1940s that poliovirus, could be cultivated in the mammalian tissue of non nervous tissue origin. Three types of tissue cultures are available. Namely the -

1. Organ culture –small bits of organs are maintained *in vitro* for cultivation of viruses preserving their original architecture and function. Example is that of the Tracheal ring organ culture for the isolation of Coronavirus.
2. Explant culture- fragments of minced tissue can be grown as 'Explants' embedded in plasma clots. Example is that of the adenoid tissue explants for the isolation of adenovirus.
3. Cell culture- tissues are dissociated into the component cells by the action of proteolytic enzymes like trypsin and the process is also aided by mechanical shaking. The cell cultures are maintained *in vitro* by the addition of the growth medium which has the following components- 10% fetal calf serum, 13 essential amino acids, 9 vitamins, salts, glucose, buffering system, antibiotics to prevent contamination and phenol red as the indicator. The cell suspension is dispensed into bottles, tubes or petridishes. The cells adhere to the glass surface and on incubation divide to form a confluent monolayer sheet of cells covering the surface within about a week. Based on their origin, chromosomal characters and the number of generations through which they can be maintained they can be either - Primary, diploid or the continuous cell cultures.

   The primary cell cultures are prepared directly from animal / human tissues, which can be subcultured only once / twice and the examples include- primary rabbit kidney cells, human amnion cells. They are

capable of only limited growth in culture and cannot be maintained in serial culture. Primary cell cultures are useful for the isolation of viruses and their cultivation for vaccine production. The diploid cell cultures are cells of single type that retain the original diploid chromosome number and karyotype during serial sub cultivation for a limited number of times. They can be prepared directly from the human fetal tissues and they can be sub cultured only upto 20- 50 times which includes MRC-5 and human diploid fibroblasts. The continuous cell cultures are derived from the tumours of animal / human tissue and main advantage is that can be subcultured any number of times. Standard cell lines that are derived from human cancers such as HeLa, Hep-2, and KB cell lines have been used worldwide for the isolation of viruses for an indefinite period of time. The viruses are cultivated by inoculating the specimens into a monolayer of cultured cells and the viruses are allowed to adsorb into the cells by shaking / rocking in room temperature for a period of one hour after which maintenance medium, which contains only 0.5-1% fetal calf serum is added and incubated (Ananthanarayan et al., 1984).

3.10.8 DETECTION OF VIRUS GROWTH IN CELL CULTURES:

The growth of virus in cell cultures can be detected by either of these methods -

1. Cytopathogenic changes
2. Metabolic inhibition
3. Hemadsorption
4. Intereference
5. Transformation
6. Immunofluorescence

Cytopathic changes – Many viruses produce morphological changes in cultured cells in which they grow. Cell cultures are typically viewed microscopically to detect any morphological changes called as the 'Cytopathic changes' and the viruses causing the CPE are called as the 'cytopathogenic viruses'. The CPE produced by different group of viruses are characteristic and help in presumptive identification of virus isolates. Adenovirus produces grape like clusters; enteroviruses produce degeneration and crenation of the cells. The time required to detect CPE varies from 1-2 days after inoculation for herpes simplex virus to 1-3 weeks for CMV. To overcome the disadvantage of the cell culture for prolonged incubation periods the rapid shell vial technique was first developed for the cultivation of CMV (Gleaves et al., 1984; Griffiths et al., 1984). The cells are grown as a monolayer above the coverslips and allowed to infect by centrifugation at 3,000 rpm for 1 hour after which the maintenance medium is added and incubated. Later the cover slips are taken and fluorescent antibody staining of the cell culture is done, regardless of whether CPE is visible. The main

advantage is that rapid detection of the viral antigen even before the production of the CPE. Metabolic inhibition - is another phenomenon where when there is viral growth the cellular metabolism is inhibited and hence the colour of medium remains unchanged. Hemadsorption - used for detecting Hemagglutinating viruses, like Influenza & parainfluenza. Guineapig erythrocytes are used which gets adsorbed onto the surface of the infected cells. Interference phenomenon- Certain viruses don't produce any cytopathic changes and hence these viruses can be detected by this technique, where the noncytopathic viruses are challenged with a cytopathic virus. The technique was first used for the detection of Rubella virus by Weller & Neva *et al* (Matsuno et al., 1984). Rubella viruses produce CPE only in primary human amnion cell cultures and no CPE is produced in African green monkey kidney cells. To describe the technique in detail for each specimen 2 tubes of AGMK cells are used, in which both the tubes are inoculated with the specimens and incubated for 10 days. One of the tubes is taken and inoculated with a challenge virus and one uninoculated tube is also inoculated simultaneously. The tubes are inoculated for at least 3- 4 days. If CPE is seen in the tube inoculated with the specimen then the virus is present and the other tube inoculated with the specimen is harvested and stored. The main disadvantage is that the delay in getting the results at least 14 – 15 days. The immunofluorescence staining can also be used for

confirming the results. Transformation- tumour forming viruses induce cell transformation and loss of contact inhibition, so that growth appears in a piled up fashion producing ‘Microtumours’. Another modification of traditional cell culture involves the use of genetically engineered cell lines. In these systems, genes are transfected into indicator cell lines to direct insertion of viral receptors on the surface of the cell and / or direct expression of promoters that respond to a specific viral protein in the specimen. Activation of the promoter triggers a reporter enzyme such as β- galactosidase that acts on a substrate to indicate the presence of the virus being sought. This approach has been widely used for HSV (Stabell et al., 1993). The main advantage of the cell culture technique is that relative ease compared to animal inoculation, increased sensitivity compared to conventional methods and recovery of unknown viruses. The main disadvantages are that inherent time delay in virus growth, maintaining cell cultures, decreased sensitivity of cell cultures at higher passage levels and the presence of inhibitory substances in the serum and the specimen as such itself. Virus isolation is seldom used for the diagnosis of post-natally acquired rubella, as serological tests are more rapid and reliable. Virus isolation plays a major role in the congenitally acquired rubella and for determining the duration of virus excretion in such infants. Virus isolation has also been employed in to

asses the risk of reinfection and the inadvertent vaccination of rubella susceptible pregnant women (Best et al., 1989).

3. 10. 9 Specimen collection:

Rubella virus can be isolated from various specimens like - Respiratory secretions, blood, urine and stool specimens in cases of postnatally acquired rubella. In cases of congenitally acquired infections the specimens collected include- Throat swab, Cataract material, lens fluid, tears, cerebrospinal fluid, autopsy material, amniotic fluid, placenta, fetal tissues incases of spontaneous and therapeutic abortions. Virus may be isolated from the pharynx 1 week before and until 2 weeks after rash onset. Care should be taken that the specimens collected for the isolation purpose should always be transported in the transport media and it should be stored at 4ºC until inoculated .If inoculation is going to be delayed then the specimen should be frozen in -70ºC (Best et al., 1989).

3. 10. 10 Processing of specimens:

The sterile body fluids can be inoculated directly into the susceptible cell cultures followed by adsorption onto the monolayer for 1-2 hours at room temperature before fresh maintenance medium is added. Cell cultures are incubated at 37ºC and medium changed twice weekly. If there is going to be tissue specimens then the tissue specimens should

be finely minced with sterile scissors and suspended in approximately four times their volume of the appropriate serum free maintenance medium. Then the sample is centrifuged at 3000 rpm for 5 minutes at 4ºC. The supernatant fluid is inoculated into the appropriate cell cultures (Best et al., 1989).

3. 10. 11 Cell cultures:

Cell cultures used for virus isolation are the following-

Primary cell cultures used for isolation include- Vervet monkey kidney cell line, Patas monkey kidney, Human amnion, Human thyroid, Human embryo kidney, Rabbit kidney, Rabbit embryo kidney. Continuous cell lines used routinely for the isolation include- Rhesus monkey kidney (LLC-MK2), Vervet monkey kidney (BSC-1), Rabbit kidney (RK13), Rabbit cornea (SIRC), Baby hamster kidney (BHK-21). Some batches of calf serum contain inhibitors that interfere with the growth of rubella virus, hence the serum used both in the growth and the maintenance media should be prescreened. Usually the specimens are initially to be inoculated into cell lines like Vero for 7-10 days. Cell cultures are then freeze thawed and passed into further Vero cultures for identification by indirect immunofluorescence staining or into RK13 or SIRC cell cultures in which the virus can be identified by their characteristic cytopathic effect of perinuclear inclusions. The cytopathic effect production in these cell cultures

occurs only if the cells are maintained under carefully controlled conditions using pretested serum. The production of CPE is also influenced by the passage number of the cells as the cells become old they become less susceptible for the viral infection (Best et al., 1989). Since this virus doesn't produce CPE in all the cell cultures the presence of the virus can be identified by the 'Interference phenomenon'. The presence of virus in the cell culture doesn't allow the growth of other virus, which produces CPE like the Coxsackie virus A9, vesicular stomatitis virus& ECHO-11. This technique was one of the first used to identify the virus in cell cultures. The main disadvantage of virus isolation is the time factor in which it takes nearly 2 -3 weeks to obtain any result.

3. 10. 12 Detection of Viral antigens:

Rubella virus antigens can be detected by flow cytometry and the viral RNA can be detected by molecular method like the reverse transcriptase polymerase chain reaction. Flow cytometry provides a rapid and effective method for fractionating the cell populations, which when used in conjunction with selected monoclonal antibodies provides a highly sensitive method for detecting antigens expressed on the cell surface. It has the potential for rapid detection of Viremia in women presenting with rubella like illness during pregnancy (Best et al., 1989).

3. 11 Serological diagnosis:

The conventional diagnosis of viral infections can be done either by direct demonstration of the antigen present in the sample or by the demonstration of the antibodies produced against these antigens. The detection of these specific antibodies is called as the serological diagnosis, which is just a supportive test and the results have to be confirmed by performing a more reliable tests like Polymerase chain reaction (PCR) which is one of the Molecular method that help in diagnosis. Serological diagnosis can be defined as the detection of the specific antiviral antibodies, whose clinical utility is limited by need for the comparison of acute & convalescent serum samples. Serological diagnosis plays an important role especially in the case of viruses that are uncultivable; determine the immune status of the individual & supports the significance of the isolated viruses (Schmidt et al., 1989). The presence of the specific antiviral IgM antibodies indicates a recent infection and it is almost a confirmatory test in case of viruses like the – Cytomegalovirus, Epstein barr, Hepatitis A, Hepatitis B, Parvovirus B19, Measles, Mumps, Rubella & Arbo viruses. The viruses infect the cells following which the primary humoral immune response is elicited, IgM antibodies are produced which stays for a stipulated period of time following which high titre of IgG antibodies are produced. In case of reinfection IgG rapidly and

specific IgM remains the same. In case of certain viruses like the Human Immunodeficiency viruses and Hepatitis C viruses the presence of antibodies indicates a chronic infection (Banatvala et al., 1984). Serology is the most common method of confirming the diagnosis of rubella. Acute rubella infection can be serologically confirmed by a significant rise in the rubella antibody titer in acute and convalescent serum samples or by the presence of rubella specific IgM. Serum samples should be collected as early as possible i.e. within 7-10 days after the onset of illness and again 14-21 days later. In cases of reinfection, rubella specific IgM is usually absent or present at a low level transiently.

The various serological tests that are available for the diagnosis include -

1. Hemagglutination inhibition test
2. Single radial hemolysis
3. Latex agglutination
4. Enzyme linked immunosorbent assay (ELISA)
5. Radioimmunoassay
6. Complement fixation
7. Neutralization &
8. Passive Hemagglutination.

3. 11. 1 HEMAGGLUTINATION INHIBITION TEST:

This test was first described by Stewart *et al* in 1967. This test is based on the principle that antiviral antibodies present in the specimen binds with the antigen forming an antigen – antibody complex and hence the antigen is not free to agglutinate the RBC that is added in the test medium. The specificity of the HAI test varies with different viruses. The sensitivity of the viral Hemagglutination inhibition tests is markedly influenced by each of the variables in the test system; these include the indicator erythrocytes, the composition of the diluents, the type and amount of the antigen, the method employed for the removal of nonspecific inhibitors from the sera, the method used for absorption of natural agglutinins from sera, and the conditions of incubation. The test can be performed only after the removal of nonspecific inhibitors like heat stable serum lipoproteins in the serum sample. The inhibitors can be removed by treatment with kaolin, heparin, $MnCl_2$. The non - specific agglutinins for erythrocytes may be removed by the addition of erythrocytes to the sera prior to testing to allow the erythrocytes to absorb the non - specific agglutinins. The RBCs used for the test should be highly sensitive to agglutination which includes baby chick, pigeon and trypsinized human O cells. The HAI test is simple to perform and requires inexpensive equipments and reagents. Briefly the procedure is as follows- The serum is pretreated to remove the nonspecific inhibitors and then doubling dilution of the serum sample

is done to which is added equal volumes of 4 - 8 HA units of rubella hemagglutinating antigen followed by incubation at room temperature for 1 hour. Then added equal volumes of 0.025%RBCs and incubated at 4℃ for 1 hour at the end of which the results are read. Advantages – The test is easy & inexpensive to perform. Disadvantages – The test is not as sensitive as the EIAs or RIAs, the actual reading of results is subjective and the test cannot be performed on all viruses (Schmidt et al., 1970; Best et al., 1989).

3. 11. 2 SINGLE RADIAL HEMOLYSIS:

The test is based on the principle of reaction between antigen & antibody which fixes the complement leading to lysis. This test is mainly used for Rubella virus antibody screening and the test is considered positive if there is going to be a zone of hemolysis greater than 15000 IU/ l. Recent infection is suggested if the serum contains HAI antibodies, but in SRH there is no zone or only a hazy zone of hemolysis. A rising titer of SRH should always be confirmed by an alternative technique or rubella specific IgM (Schmidt et al., 1989).

3. 11. 3 LATEX AGGLUTINATION:

The test is based on the principle of antigen – antibody reaction which is made visible to the naked eye by the presence of the latex particles. The carrier particles are the latex beads which are coated with the

specific antigen, which reacts with the antibody and the agglutination read macroscopically (Schmidt et al., 1989).

3. 11. 4 ENZYME IMMUNOASSAY:

Enzyme labelled conjugates were first introduced in 1966 for localization of antigens in tissues, as an alternative for fluorescent conjugates. In 1971, enzyme labelled antigens and antibodies were developed as serological reagents for assay of antigens and antibodies. The term Enzyme immunoassay includes all assays based on the measurement of enzyme labelled antigen, hapten or antibody. Basically there are two types of enzyme immunoassays- Homogenous and Heterogenous. The homogenous EIA does not involve the separation of the bound and free fractions and hence the test can be completed in a single step.

The heterogenous EIA requires the separation and multiple steps are involved. The major type of heterogenous EIA is the Enzyme Linked Immnuosorbent Assay (ELISA). There are various methods – Competitive ELISA, Non competitive ELISA, Sandwich ELISA, antibody capture ELISA and Cassette ELISA (Ananthanarayan et al., 1996; Matter et al., 1994). The most commonly preferred test for rubella diagnosis is the ELISA test for IgM and IgG antibodies.

3. 11. 5 Competitive ELISA:

In this system one of the components of the immune reaction is insolubilized and the other one is labelled with the enzyme. The analyte can then be quantified by its ability to prevent the formation of the complex between the insolubilized and the labelled reagent.

3. 11. 6 Non competitive ELISA:

The serum antibody binds with the respective antigen and the presence of the complex is detected by the addition of the anti- antibody labelled with the enzyme.

3. 11. 7 Sandwich ELISA:

Here the same component of the immune reaction is used in the insolubilized and the enzyme labelled form. The other component, the analyte (i. e. the antigen in the sample forms a bridge between the two reagents). The method in which one component is used in a insolubilized form to bind the analyte from the sample, which is subsequently determined by the addition of the labelled second antibody against the same class of antibody as the analyte antibody. In principle, quantification can be achieved over an extremely wide analyte concentration range in such sandwich methods.

3. 11. 8 Antibody capture ELISA methods:

These methods used to detect antibodies of specific immunoglobulin subclasses, by first allowing the sample to react with e.g. insolubilized anti- IgM and subsequently with either enzyme labelled antigen followed by enzyme linked antibody. Advantages- very specific, no interference with rheumatoid factor, used specifically for detecting IgG and IgA subclasses.

3. 11. 9 ELISA – IgG Avidity test:

A further refinement of the ELISA test is the urea avidity test, which is performed on the IgG antibodies. In those pregnant mothers who test IgM positive the avidity test distinguishes primary from secondary / reactivation rubella infection. The principle of the test is based on the increasing avidity that IgG antibodies display for their specific antigen with increasing time after infection. Thus IgG antibodies produced in the primary infection have a considerably lower avidity than those produced in the reactivation / reinfection. The avidity differentiation may be demonstrated by incorporating a protein-denaturing agent, usually urea, into the washing fluid in the ELISA test. Primary antibodies are far easier to detach from their antigenic sites than are reactivation antibodies during the ELISA washing process. The difference in the final optical density reading, before and after urea washing is expressed as an avidity index. Thus in the primary infection

IgG antibodies usually have an avidity index of less than 30%, while those produced in reactivation / reinfection are usually greater than 70% (Schmidt et al., 1989).

3. 11. 10 Detection of Rubella specific IgM antibodies:

The commonly used methods for the detection of rubella specific IgM include the Radioimmunoassay, Enzyme immunoassay and some laboratories also use the solid- phase hemadsorption –inhibition test employing RBCs for the detection of bound RV antigen. The most sensitive and reliable technique for routine diagnosis of postnatal and congenital infection are the M-antibody capture radioimmunoassay (MACRIA) and M – antibody capture enzyme immunoassay (MACEIA). Both these techniques exploit the availability of labeled monoclonal antibodies for the detection of rubella virus antigen (Matter et al., 1994; Best et al., 1989).

3.12 RADIOIMMUNOASSAY:

This method is the same as that of the enzyme immuno assay in which instead of the fluorescent dyes the Radiolabels is used. A variety of tests have been devised for the measurement of antigen & antibodies using the labelled reagents. The substance whose concentration is to be determined is termed as the ‘Analyte/ Ligand’. The binding protein, which binds to the ligand, is called the ‘binder’. RIA is a competitive

binding assay in which the fixed amount of antibody and the radiolabelled antigen react in the presence of unlabelled antigen. The labelled and unlabelled antigens compete for the limited binding sites on the antibody. This competition is determined by the level of unlabelled antigen present in the reacting system. Once the reaction is over the antigens are separated into free and bound fractions and their radioactive counts measured. Using a dose response curve the concentration of test antigen can be calculated from the ratio of total and bound antigen labels (Ananthanarayan et al., 1984).

3. 13 COMPLEMENT FIXATION TEST:

The complement fixation test was extensively used in the syphilis serology after being introduced by Wasserman *et al* in 1909. The test is convenient and rapid to perform; the demand on equipments and reagents is small and a large variety of test antigens are readily available. In essence the test consists of two antigen – antibody reactions, one of which is the indicator system. The first reaction takes place between the known virus antigen and the specific antibody in the presence of predetermine amount of complement. The complement is removed or “fixed” by the antigen – antibody complex. The second antigen antibody reaction consists of reacting sheep RBC with hemolysin. When this indicator system is added to the reactants, the sensitized RBCs will only lyse in the presence of free complement.

The antigens used for CFT are only Group antigens rather than type specific antigens. Advantages – Ability to screen against a large number of viral infections at the same time. Disadvantages- Not sensitive cannot be used for immunity screening, time consuming, labor intensive & often nonspecific reactions are seen (Best et al., 1989).

3. 14 NEUTRALIZATION TEST:

Neutralization of a virus can be defined as the loss of infectivity through reaction of the virus with specific antibody. Virus and serum are mixed under appropriate condition and then inoculated into cell culture, eggs or susceptible lab animals. The presence of unneutralized virus can be detected by reactions such as CPE, hemadsorption / hemagglutination, plaque formation, disease in animals. There are two types of Neutralization namely – Reversible and stable neutralization reactions.

Reversible Neutralization – The neutralization reaction can be reversed by diluting the antigen – antibody mixture within a short time of formation of the complex. It is due to the interference with the attachment of virions to the cellular receptors eg. The attachment of the HA protein of the influenza virus to the sialic acid. The process requires the saturation of the surface of the virus with the antibodies.

Stable Neutralization - The antigen antibody complex that has formed becomes more stable with time and hence the processed cannot be reversed by dilution. Neither the virions nor the antibodies are permanently changed in stable neutralization, for the unchanged components cannot be recovered. It has been shown that neutralized virus can attach and that already attached virions can be neutralized. The number of antibody molecules required for stable neutralization is considerably smaller than that of reversible neutralization. An example of stable neutralization is neutralization of the poliovirus, whereby; the attachment of the antibody to the viral capsid stabilizes the capsid and inhibits the uncoating and release of viral nucleic acid (Schmidt et al., 1989).

3. 15 MOLECULAR TECHNIQUES:

The detection of viral genome (that is, viral nucleic acid, either DNA or RNA) has gained enormous importance over the last decade in the diagnosis and management of viral infections and diseases. Nucleic acid testing has proven its superiority over more conventional laboratory techniques in several areas for example:

- For viruses that cannot be cultured in the conventional cell culture system
- For clinical sample of small volume
- Where antibody seroconversion is delayed after an acute infection

- ❖ In the immunocompromised patients who might have a suboptimal antibody response.
- ❖ For the diagnosis of congenitally or perinatally acquired viral infections.

Principally the molecular biological assays that test for nucleic acid consists of three components:

Front end - Sample preparation, often consists of extracting and purifying the nucleic acids from the clinical material.

Middle part- Hybridization to the target nucleic acid sequence or the genome amplification techniques where there is amplification of the target sequences.

Back end - detection & if possible quantification of the nucleic acid sequences (Read et al., 2000).

Front-end techniques:

The objective that must be borne in mind is the efficient removal of the DNA or the RNA from other inhibitors or contaminating substances from the sample. The front end must be chosen according to the type of clinical specimen from which the nucleic acids are going to be recovered, its quantity & quality and the type of nucleic acids that is going to be detected. Although there are various methods of DNA extractions including the conventional Phenol chloroform methods, the trend is towards the faster and more easily handled

methods like the nucleic acid binding to the silica membrane (Preiser et al., 2000).

Middle part techniques:

Once the nucleic acid is extracted different techniques are available for the detection and quantification of the target DNA / RNA. The detection techniques can be broadly classified into -

- Target amplification techniques - eg include the Polymerase chain reaction & nucleic acid sequence based amplification techniques (NASBA)
- Signal amplification techniques - eg include the branched DNA assays and Hybridization assays.

3. 15. 1 Target amplification techniques:

This is based on the principle of enzymatic amplification of the target sequence thereby increasing the target sequence copies in the sample.

The most widely used technique is the Polymerase chain reaction (PCR) invented by Kary Mullis and described first by Saiki *et al* has received wide spread application in many diverse fields of medicine. There are many modifications of PCR, which includes Reverse transcriptase polymerase chain reaction (RT-PCR), Nested PCR, Inverse PCR, Multiplex PCR. The quantitation of the nucleic acids can be done by the quantitative PCR (Preiser et al., 2000).

3. 15. 1 Polymerase Chain Reaction:

The PCR is a technique that enables the amplification of the specific sequences of nucleic acids. Khorana and collegues have described this technique over a decade earlier and this technique has received wide spread application in many diverse fields such as forensic science, histopathology and prenatal diagnostics (Baumforth et al., 1999; Mullis et al., 1986; Saiki et al., 1985; Kleppe et al., 1971).

Basic Principle of PCR:

In the PCR, two primers, which are short single stranded DNAs are used that are complementary to opposite strands of DNA sequences to be amplified. After the heat-mediated denaturation of the template DNA, the primers anneal to their respective sequences on the template DNA and a DNA Polymerase synthesizes a complementary strand in the 5’ to 3’ direction, extension. Each round of denaturation, annealing and extension is known as a cycle. Theoretically, with each cycle the amount of the template DNA sequence amplified doubles. However, an “amplification plateau” will eventually be reached when additional cycles will not lead to any further increase in the amplified product. This amplification plateau results from the exhaustion of reagents such as dNTPs and primers. The optimum temperature at which each of these steps – denaturation, annealing and extension proceeds is different and therefore the reaction is best performed in a thermal

cycler, which automatically makes the temperature changes required (Baumforth et al., 1999).

Primer design and optimization:

Successful specific amplification of the desired target sequence in the PCR is dependent upon both the design and optimal use of the primer pair. Innis et al., 1990; Kocher et al., 1991; Old et al., 1994; Taylor et al., 1992; Rybicki et al., 1994 have set forward some simple set of rules for the design of PCR primers, namely-

- ✓ Primers should range from 15 – 30 bases in length.
- ✓ Base composition should be 50 –60% guanine + cytosine.
- ✓ Long runs with more than three or four of the same base should be avoided.
- ✓ Primers should not have secondary structures for example- hairpin loops.
- ✓ Ideally, primers should not contain sequences that are complementary to each other. This will avoid the annealing of primers to each other – primer dimmer formation.
- ✓ Palindromic sequences should be avoided.
- ✓ Primer melting temperatures (T_m) between 55 and 80°C.

  Ideally, the primer pair should show no homology with unwanted sequences within the sample and, for this reason, primer sequences

should be checked for any complementarity to other known DNA sequences by using a sequence database (Baumforth et al., 1999).

Reaction Components:

DNA Polymerase:

The most commonly used DNA Polymerase is *Taq* Polymerase, which is obtained from a bacterium found in hot springs known as *Thermus aquaticus*, and is available commercially. *Taq* Polymerase works optimally at 72°C and over the pH range 7.0 – 7.5, adding approximately 100 nucleotides / second to the primer (Kocher et al., 1991). *Taq* Polymerase is also heat stable, allowing the enzyme to withstand repeated denaturation cycles. One drawback of *Taq* Polymerase is that it lacks 3'→ 5' exonuclease, proof reading activity which can lead to misincorporation of nucleotides (Gelfand et al., 1992; Eckert et al., 1992).

Deoxynucleotide triphosphates:

dNTPs are available commercially either as freeze dried or neutralized aqueous solutions. They are also available as either radioactively labelled or non radioactively labeled and this is used mainly in hybridization or sequencing of PCR products. These are heat resistant and have a half life of more than 40

cycles of PCR. It is used at a concentration range of 50µM to 200µM (Baumforth et al., 1999).

Reaction Buffer:

The buffer most often used in the PCR is 10mM Tris buffer, with a pH range between 8.5 and 9.0 at 25°C. Because the pH of Tris buffers decreases by 0.3 units for each 10°C rise in temperature, a buffer made to pH 8.8 at 25°C will have a pH value of 7.4 at 72°C, the optimal value for the activity of *Taq* Polymerase. The appropriate concentration of magnesium ions, the reaction buffer is also important for maximal Taq Polymerase activity and, because the dNTPs bind magnesium ions, the reaction mixture must contain an excess of magnesium ions. The magnesium concentration in the reaction mixture is generally 0.5 – 2.5 mM greater than the concentration of dNTPs.

Primers:

Primer concentration should not be greater than 1 µM. High primer concentrations promote mis priming, the formation of primer dimmers, or the generation of non-specific products, thereby reducing the yield of desired product (Innis et al., 1990). The optimal concentration of primer pairs must be ascertained for a given PCR system by titration. As a general rule lower

concentration of primers should be used with increasing concentrations of template or increasing template complexity. Limited mismatches at 5' end do not significantly affect the efficiency of amplification, those at the 3' end will prevent extension by the DNA Polymerase.

Template DNA:

For a successful PCR the sequence to be amplified must be intact. Poor quality DNA, will often contain short DNA fragments. In such cases, primers should be designed to amplify shorter regions within the template DNA. Low concentrations of DNA are required for optimal PCR because both primers and dNTPs should be in excess; an overabundance of template DNA will favour annealing of the two strands of the template sequence, rather than their annealing to the primer pair, and will also increase the chances of forming non specific products (Innis et al., 1990).

Avoiding contamination in the PCR:

Contamination of the sample with an exogenous sequence at any stage from sample preparation to the reaction itself can result in the amplification of this contaminant sequence and hence a false positive result. Since PCR is very sensitive, contamination with

even extremely small quantities of exogenous material can result in amplification. The contamination might be of laboratory origin or of external origin. Hence to minimize the risk of contamination the PCR stages namely, Pre PCR i.e. the sample preparation, setting up of the PCR reaction and analysis of the results should be done in separate rooms. All consumables should be autoclaved before use. Operator contamination can be reduced by wearing suitable protective clothing like gloves (Kwok et al., 1990).

Alternate target amplification techniques:

Ligase chain reaction (LCR) developed by Abbott laboratories uses a thermostable ligase to join two oligonucleotides to each other when they are hybridized to their respective target sequences. Amplification of the nucleic acid sequences by LCR is exponential because each round of synthesis can double the number of target molecules (Read et al., 2000).

3. 15. 2 MULTIPLEX PCR:

In diagnostic laboratories the use of PCR is limited by cost and sometimes availability of adequate test sample. To overcome these shortcomings and also to increase the diagnostic capacity of PCR, a variant termed multiplex PCR has been described. This is a

modification of the standard PCR wherein two or more primer sets are added into the same tube to help in the detection of 2 or more organisms. Development of multiplex PCRs should follow a rational approach for the inclusion or exclusion of specific pathogens in the assay. These pathogens can be organ system specific or symptom specific and the epidemiological characteristics of these pathogens.

Co - amplification of different target serves several purposes namely-

- Large regions of the DNA sequence can be scanned for alterations
- Unrelated segments of the target genomes can be tested
- Internal controls for the amplifiability of the sample can be included
- Cost-effective panels of tests for multiple pathogens from a single specimen can be developed.

The standardization of this technique is very cumbersome but once standardized it is very useful in the rapid identification of the causative organisms in a single tube reaction. The amplified products size should differ at least by 30 bases for the easy visualization of the products on the agarose gel (Elnifro et al., 2000; Preiser et al., 2000; Baumforth et al., 2000).

3. 15. 3 POINT MUTATION DETECTION:

The amplification refractory mutation system (ARMS) uses PCR primers with 3' mismatches and a polymerase that lacks 3' to 5' exonuclease activity to detect specific alterations in a nucleotide sequence. The enzymatic extension of an oligonucleotide primer depends on stable base pairing of the 3' end of an oligonucleotide. When a mismatch occurs, primer extension is often prevented. The ARMS uses selective amplification based on 3' mismatches to identify specific mutations by demonstrating the presence of a nucleotide sequence that cannot be amplified with primers containing the expected mutations (Persing et al.,2000).

3. 15. 4 NESTED PCR:

The sensitivity of PCR can be affected adversely by the presence of low copy template nucleic acid. Both the sensitivity and specificity of the PCR can be increased by using a nested PCR. In nested PCR (nPCR), two separate amplifications are used. The first sets of primers are used for the amplification of the target genome. This step produces an amplification product, which is transferred to a new reaction tube for a second round of amplification by using a second primer pair that is specific for the internal sequence amplified by the first primer pair. The second amplification usually proceeds for an additional 15 to 30 cycles, after which the products

are detected by gel electrophoresis. nPCR increases the sensitivity, often a single copy of the target can be detected without the need for hybridization. Reamplification with the second set of internal primers also serves to verify the specificity of the first round product. In addition, the transfer of reaction products from the first reaction effectively serves to dilute out inhibitors that might be present in the sample initially (Bamuforth et al., 1999).

3. 15. 5 HOT PCR:

Variation of the nPCR is that in hot PCR an end labelled primer is used in the second amplification step. End labelling of the primer is performed before the reaction, by incubating the primer with polynucleotide kinase and [γ^{35}S] ATP. After the second amplification the products are run on a PAGE, followed by auto radiography (Bamuforth et al., 1999).

3. 15. 6 INVERSE PCR:

In standard PCR, a known sequence is amplified between the primer annealing sites. However it may be desirable to amplify the sequences on either or both sides of the known region. This can be achieved by inverse PCR. The intial stage is the digestion of the DNA with the restriction enzyme. The restriction enzyme should be chosen in such a way that it cuts the DNA outside the known

sequence. The digestion is followed by the ligation of the restricted fragments. A second restriction enzyme is used for the linearization of the circular DNA fragments. This cuts the DNA within the known sequence, producing a linear DNA fragment in which two halves of the known sequences are situated at either end. The unknown sequence is now amplified using the standard PCR (Bamuforth et al., 1999).

3. 15. 7 DNA SEQUENCING:

Sequencing of the genome has the potential to distinguish between parent and progeny if a single mutation has occurred in the replicative process (Arens et al., 1999). DNA sequencing using the PCR follows the procedure that is very similar to standard PCR, except that the dNTPs are labeled with either a radioactive or fluorescent tag. In case of radioactive labeling, four reaction tubes are prepared, each of which contains template. Primers, *Taq* Polymerase, all four dNTPs (one of which is labeled), and one of the four dideoxy (dd)NTPs. Fluorescent dye labelling is the commonly used one which can be carried out in a single tube with all the components of PCR added and the 4 dNTPs each labelled with a different fluorescent dye since each of the four dye fluoresces at different wave length (Howe et al., 1995). Two chemistries have been proposed and they are-

Dye termination- any primer can be used along with four fluorescently labelled ddNTPs, it is easier and it is able to cope up with the single or double stranded DNA. However, the specificity of the *Taq* Polymerase results in uneven incorporation of the labeled bases. Dye primer- Here the primer used for the chain extension is prelabelled with the fluorescent dyes. However, dye primers are expensive to synthesize and are available only of the commonly used primers such as T7, T3, M13, etc., Therefore, before sequencing, the template has to be cloned into one of these cloning vectors. Irrespective of the labelling method used the sequencing of the product begins when the DNA strands are separated and a single primer is allowed to anneal. The extension reaction takes place in the presence of the dNTPs and either one of the labelled ddNTPs. The ddNTPs act as extension terminators. The DNA sequences produced by the PCR reaction will share a common 5' end but have different 3' ends, depending upon which ddNTP halted extension. The various lengths of DNA are then separated by size using PAGE and detected by autoradiography or detected directly using the laser beam (Preiser et al., 2000).

3.15.8 REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION:

Reverse transcription polymerase chain reaction is the most sensitive and versatile method developed so far for the analysis of gene expression in cells and tissues. It relies on the initial conversion of RNA to complementary DNA (cDNA) using a reverse Transcriptase enzyme. Most reverse transcriptases used so far have been isolated from viruses – for example, the avian myeloblastosis virus (AMV) and the Moloney murine leukemia virus (MMLV). Both the enzymes work well, but AMV reverse Transcriptase has the advantage that the optimum temperature for reverse transcription is 42°C, which is of benefit if the RNA template has a high degree of secondary structure. The single stranded cDNA produced by the RT reaction is then amplified during the first cycle of the standard PCR by *Taq Polymerase* to yield double stranded cDNA, which is then amplified in further cycles. Hence RT-PCR can also be used to obtain cDNA for sequence determination and subsequent cloning without having to resort to constructing and screening a cDNA library. There are various strategies for obtaining the first strand cDNA using the reverse transcriptase reaction; either by a downstream (antisense) PCR primer annealed to the RNA, by the use of random hexamers, or by an oligo d (T) primer at the poly(A) tail of mRNA (Kawasaki

et al., 1990). Each of these approaches has its drawbacks. For example, oligo d(T) primers that anneal to the extreme 3' end of the transcript might not efficiently synthesize cDNA from long mRNAs or those with secondary structure. The use of an antisense primer limits the subsequent PCR to a single product and will, therefore, necessitate the use of paired sense and antisense primers, whereas random hexamers will permit the cDNA template to be used for a number of independent PCRs. RT-PCR is used to detect viruses with an RNA genome and to detect RNA transcripts. Various methods of RNA extractions are available and the extracted RNA should be carefully stored free from RNAse. Because of the sensitivity of RT-PCR, total cellular nucleic acid can be used, without the need to isolate poly(A) mRNA. However, cellular DNA will also be present in such preparations and amplification of this endogenous DNA is avoided by previous treatment of the sample with deoxyribonuclease. For RT-PCR, the cDNA produced can also be validated by amplifying housekeeping genes that span single or multiple introns. Consequently, this system permits both verification of the cDNA template and identification of contaminating DNA (Nelson et al., 1996). In addition to the controls used in the standard PCR, RT- PCR should also include a control in which RT enzyme is omitted. This also ensures that the RNA preparation does not contain residual

contaminating DNA. Advantages of RT-PCR- highly sensitivity and specificity when gene specific primers are used for cDNA synthesis.

Disadvantages of RT-PCR is RNAse contamination leading to false negative results. It is relatively an expensive procedure (Innis et al., 1990).

3. 15. 9 Competitor RNA in Reverse Transcription PCR:

Competitor RNA can be prepared by restriction enzyme digestion of the cDNA product at two restriction endonuclease cleavage sites, which generates compatible ends; this is followed by religation (Kozbor et al., 1993). Although this approach is straight forward, synthesis of competitor RNA by this method requires cloning of template DNA into a plasmid vector and is dependent on the presence of two unique restriction endonuclease cleavage sites within the intervening sequence. Additionally, for this method the RNA Polymerase promoter site must be incorporated after the deletion is made, either through the addition of a 5' extension to one of the oligonucleotide primers or through the inserting of the RNA Polymerase promoter site into a plasmid vector that contains an RNA promoter. An additional method for competitor RNA synthesis uses an RNA ligase mediated approach (Borriello et al., 1995). However, this approach requires multiplePCRs, which add

both time and complexity to the protocol. Other method of competitor RNA production is by synthesis of “armored” RNA, in which RNA is encapsidated by bacteriophage coat proteins and thus protected from degradation by RNases (Pasloske et al., 1998). Competitor RNA transcribed *in vitro* can be used for several applications in diagnostic RT-PCR. Use of competitor RNA as an extraction control allows the rapid, comprehensive assessment of both the RNA recovery efficiency and the exclusion of RNase enzymes from the extraction procedure. Additionally competitor RNA can serve as positive control template for validation and use of RT-PCR assays. When competitor RNA is used as a positive control, false positive reactions caused by cross contamination with the positive control sample are readily identified due to different size of the amplicon generated from the competitor RNA. Competitor RNA can also be particularly useful as a positive control template for pathogens that are difficult to cultivate and quantify *in vitro* such as Hepatitis C virus (Kleiboeker et al., 2003).

3. 16 QUANTITATIVE PCR:

The nucleic acid present in the sample can be quantitated by a variety of methods-

Limiting end point dilutions of the PCR products

Amplification with external standards

Amplification with internal standards

Amplification using a combination of internal and external standards

Competitive amplification methods

Quantification using the Limiting dilution measures the amount of target DNA present in the sample by subjecting it to different dilutions of PCR and then detecting the presence of amplicons by electrophoresis on ethidium bromide stained agarose gel. Quantification can also be done by running the internal standards in the same tube along with the controls and in case of the external standards they are run in the same profile along with the test sample but in a different tube. Competitive PCR is by comparing the unknown target with the known amount of the competitive template. The recent advances in the field of the quantitative PCR are that of the light cycler and the Real time PCR. Light cycler is based on the theory of DNA binding with the dyes and the amount of fluorescence emitted is directly proportional to the amount of DNA present in the sample (Preiser et al., 2000; Gao et al., 1997).

3. 16. 1 REAL -TIME PCR:

In case of the Real time PCR the detection and quantification of the amplicons starts as the amplification progressed. More than five chemistries have been described and they are-

- DNA binding fluorophores
- 5'endonuclease activity
- Adjacent linear oligoprobes
- Hairpin oligoprobes
- Self fluorescing amplicons

The most commonly used one is the 5'endonuclease activity. Here the first step as usually the denaturation of the double stranded DNA into two single stranded DNA following which a non-extendable hybridization probe is added. The characteristics of the probe is that it has a reporter fluorescent dye on the 5' end and a quencher dye on the 3' end, thereby preventing the detection of the fluorescent probe. The probes are designed in such a way to bind internal to the PCR product. During the annealing process the hybridization probe binds to the single stranded DNA. Now the *Taq* DNA polymerase that is added in the system starts the synthesis of a new strand of DNA by the addition of nucleotide bases. The *Taq* DNA polymerase has 5' to 3' exonuclease activity that allows it to cleave the terminal nucleotides of double stranded DNA. Since the probe is non-extendable, the exonuclease activity removes the nucleotide from the template DNA. As the probe is degraded the quencher is separated from the reporter fluorescent dye allowing the detection of the dye. Real time PCR is preferred

over conventional PCR for routine diagnostic applications, because it is performed in a closed system and does not require post amplification manipulation of the sample. This significantly reduces the risk for carry over contamination, and eliminates the time-consuming amplicon detection step. In addition Real time PCRs have demonstrated equivalent sensitivities to conventional PCR systems, a high degree of reproducibility and a superior turn around time (Stranska et al., 2004; Abe et al., 1999; Locatelli et al., 2000; Ryncarz et al., 1999; van Elden et al., 2001).

Advantages- very sensitive and specific, the assay is quantitative and there is no post- PCR processing required. Disadvantages- cross contamination can easily lead to false positive results and the technique is very expensive (Mackay et al., 2002; Niesters et al., 2001).

3.17 NUCLEIC ACID SEQUENCE BASED AMPLIFICATION:

This is an isothermal RNA amplification reaction which makes use of three different enzymes which are active at 41℃. The process makes use of two primers, which are short single- stranded DNA fragments, and three enzymes. AMV-RT (Avian Myeloblastosis virus) containing a T7RNA polymerase promoter sequence anneals to the target and RNA: DNA hybrid is formed. The RNA strand is

then degraded by the action of RNAse H and a second DNA strand is synthesized in the presence of T7RNA polymerase. This then enters the amplification stage and the product is detected by chemiluminescent detector (Leone et al., 1998; Preiser et al., 2000).

3.18 TRANSCRIPTION BASED AMPLIFICATION SYSTEMS:

The first non-PCR target amplification (TAS) system is based on amplification by *in vitro* transcription. Each cycle of TAS consists of two parts - synthesis of DNA molecule that is complementary to the target nucleic acid, and *in vitro* transcription with the newly synthesized cDNA as the template. The synthesis of cDNA is primed by specially designed oligonucleotide primers. One end of the primer consists of a target specific sequence, and the other end contains a promoter for phage T7 RNA Polymerase. After the heat denaturation of the RNA-DNA or DNA - DNA duplexes, a second strand is synthesized by newly added reverse transcriptase; the resulting double- stranded DNA copy of the target contains a T7 promoter at one or both ends. The newly synthesized cDNA serves as a template for the synthesis by T7 polymerase of a large molar excess of RNA, which can then be used as the substrate for the next cycle of TAS (Persing et al., 2000).

3. 19 MOLECULAR BEACONS:

Molecular beacons are hair pin shaped oligonucleotides. The stem of beacon is formed by complementary sequences at both ends of the oligonucleotides. A fluorescent label and a quenching group are attached at the two ends of the molecule. The stem holds these two groups in close proximity to each other, causing the fluorescence of the fluorophores to be quenched by energy transfer. When the molecular beacon encounters a target molecule containing a sequence that is complementary to the loop sequence, a hybrid is formed. This hybridization forces the stem apart and causes the fluorophores and the quencher to move away from each other. The fluorescent signal is generated that is not quenched and the signal is read (Jos et al., 2002).

3. 20 MOLECULAR BEACONS WITH NASBA:

The isothermal amplification method for nucleic acids, NASBA, is a technology with the potential for broad applications in the field of RNA amplification and detection. With respect to other amplification systems such as the PCR techniques, the ability of NASBA to homogenously and isothermally amplify RNA analytes extends its application range from viral diagnostics to the indication of biological activities such as gene expression and cell

viability. A NASBA reaction is based on the concurrent activity of AMV reverse transcriptase (RT) (Leone et al., 1998).

3. 21 STRAND DISPLACEMENT AMPLIFICATION:

Strand displacement amplification is an isothermal, *in vitro* nucleic acid amplification technique based upon the ability of *HincII* to nick the unmodified strand of a hemiphosphorothioate form of its recognition site, and the ability of exonuclease deficient klenow to extend the 3'end at the nick and displace the downstream DNA strand. Exponential amplification results from coupling sense and antisense reactions in which strand displaced from a sense reaction serve as target for an antisense reaction and vice versa. This basic protocol of amplification has the draw back of requiring a restriction enzyme cleavage of DNA sample prior to amplification in order to generate an amplifiable target fragment with defined 5' and 3' ends. Hence several modifications have taken place to simplify the strand displacement amplification wherein it is now performed in three simple steps –

1. The target DNA sample is heat denatured in the presence of primers and other reagents.
2. *HincII* and exo$^-$ klenow are added
3. The sample is incubated at 37° C (Walker et al., 1992).

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3. 22 PROBE BASED AMPLIFICATION METHODS:

An alternative strategy for detecting rare target molecules is to amplify the probe molecule itself. Unlike target amplification methods, in which target sequences are copied into the molecule that is eventually detected, the end product of the reaction is an amplified version of the original components used to detect the target (Persing et al 2000).

3.22. 1 Qβ Replicase amplification of RNA probe molecules:

Qβ Replicase is a 215- kDa RNA directed RNA polymerase that replicates the genomic RNA of bacteriophage Qβ. This unique multiple subunit enzyme specifically recognizes a unique folded RNA structure formed by intramolecular base pairing of the Qβ RNA genome. After the probe has annealed specifically to its target and the unbound or nonspecifically bound material is washed away, the probe is enzymatically replicated *in vitro* to levels that can be readily detected (Persing et al., 2000).

3. 23 SIGNAL AMPLIFICATION METHODS:

For the purpose of increasing the sensitivity of hybridization – based tools, an alternative to enzymatic duplication or ligation of nucleic acids is to increase the signal generated from the probe molecule itself. An increase in the signal generated from nucleic acid probed is theoretically possible if the reporter group can be attached in greater numbers to the probe molecule or if the signal intensity generated by each reporter is increased.

They can be classified broadly under 2 subheads as - Hybridization and branched DNA assays. Hybridization procedures are based on the detection of a single stranded nucleic acid sequence by means of a labelled nucleic acid probe. This probe specifically binds to the target nucleic acid. The binding is later visualized depending on the nature of the label. Hybridization can be either liquid phase or the solid phase. In the solid phase the nucleic acid is bound to the solid phase and probe is used for identification. In case of the liquid the nucleic acid and the probe are free to interact with each other (Preiser et al., 2000).

3. 23. 1 Southern Blotting and Hybridization:

This is the original method of blotting invented by Ed Southern (Southern et al., 1975; Southern et al., 1979). After electrophoresis, the agarose gel containing the PCR products is placed on a wick made from the filter paper, which is in contact with a reservoir of "transfer" buffer (usually sodium citrate and sodium chloride (SSC)). The gel is then sandwiched between the wick and a hybridization membrane. Additional filter paper layers, a pad of paper tissue, glass plate, and a weight are then placed on top of the membrane. The transfer buffer is drawn upwards by capillary action through gel, removing the DNA and immobilizing it on the membrane. Nylon membranes have replaced nitrocellulose membranes as they have greater binding capacity and are less fragile. The nucleic acid can be denatured either before or after

transfer, this ensures the presence of single stranded DNA that is amenable to probing. The next step involves fixing the nucleic acids to the membrane and this is achieved by heat treatment at 80°C, or by UV cross-linking. The membrane is then placed in a solution containing labelled (radioactive or non- radioactive) nucleic acids, known as probe, which are complementary sequences on the membrane. Hybridization conditions are chosen that maximize specific binding but minimize background caused by non – specific binding. The position of the bound probe can then be visualized based on the labeling for example chemiluminescence or autoradiography. Hybridization should enable precise identification of target bands of interest.

In case of Southern hybridization it is the DNA that is used and in Northern it is RNA. In case of the RNA it should be run on a denaturing agarose gel electrophoresis and then blotted onto the membrane (Preiser et al., 2000).

3. 24 ENTEROVIRUSES:

The enteroviruses are among the most common and important viral pathogens of the humans. Enteroviruses are small single stranded RNA viruses that comprise 64 serotypes recently redistributed into five species. Poliovirus 1- 3 serotypes, Coxsackie A viruses 1 – 22 serotypes and 24 variant, coxsackie B viruses 1-6 serotypes, ECHO viruses 1-9, 11- 27, 29 –31 and numbered EVs from 68 – 72.

Infections with EVs have a wide range of clinical outcomes such as poliomyelitis, aseptic meningitis, myocarditis and acute hemorrhagic conjunctivitis particularly due to coxsackie A24 and EV70. Because of these protean clinical manifestations and serotypes, the specific microbiologic detection of a virus is very important (Park et al., 2005). Traditionally, enteroviruses have been detected by isolation in cell culture, and their serotype identity has been established by neutralization of infectivity with serotype specific antisera. These methods are labor intensive and time consuming and they require large pools of reference antisera. Moreover new strains of viruses are untypeable and negative cell culture results for clinical specimens are nondiagnostic (Muir et al., 1998; Oberste et al., 2000). These disadvantages of conventional methods have resulted in the development of molecular biology based methodologies as non-culture based methods for virus detection, particularly nucleic acid amplification methods such as PCR (Muir et al., 1998). The enzymatic amplification of single genes or short sequences of DNA by repeated cycling of Polymerase chain reaction elongation reactions has been reported by Mullis et al., 1987; Saiki et al., 1985. Comparison of published EV genome sequences has allowed highly conserved sequences to be identified. The VP1 region of the EV gene contains a major neutralization epitope, and its sequence is known to correlate well with the classical serotypes (Oberste et al., 2003). Studies by Oberste et al., in 2003 has shown that sequencing of the VP1 region

by using primers containing deoxyinosine and mixed bases for codon degeneracy could identify almost all serotypes. These sequences have been exploited as primer recognition sequences for Reverse Transcriptase PCR (RT-PCR) assays capable of detecting most or all EVs, including those, which cannot readily be propogated in cell culture (Chapman et al., 1990; Hyypia et al., 1989; Kammerer et al., 1994). Results can be obtained in as little as five hours and many therefore have a greater impact on clinical decision-making (Rotbart et al., 1994). A limitation of most PCR methods described thus far for the diagnosis of EV infections is the inability to perform serotyping or other subclassification of EVs. As a alternative approach nucleotide sequence analysis of PCR products may allow the typing of viruses detected only by PCR (Clewley et al., 1995). Studies by Khan et al., 1994; Arola et al., 1996; Muir et al., 1996a, Muir et al., 1996b have serotyped by comparing the nucleotide sequence of EV PCR products with published EV sequences to type or classify EV in the absence of a virus isolate. Other methods include RFLP analysis of PCR products (Balanant et al., 1991; Kammerer et al., 1994; Nicholson et al., 1994) hybridization with type specific probes (Chapman et al., 1990; Da Silva et al., 1991) or SSCP analysis of PCR products (Fujioka et al., 1995). Phylogenetic analysis of the virus isolates provides the necessary information for molecular epidemiological studies. Genetic differences between strains may be demonstrated by oligonucleotide mapping of viral RNA by PCR

– RFLP or more commonly by nucleotide sequence determination of PCR products, which provides the most detailed and informative discrimination between strains. Phylogentic analysis has been used for determining the date of emergence of newly arising EV serotypes or strains (Miyamura et al., 1990; Miyamura et al., 1986) and to study their subsequent molecular evolution and global spread and to confirm the common source of isolates from a single EV outbreak (Diedrich et al., 1995; Drebot et al., 1994) and study their genetic relationship to strains from previous outbreaks and to identify co circulating strains of a single serotype.

3. 25 CELL CULTURE:

Although virus isolation has been regarded as the gold standard for EV identification, virus isolation procedures are only poorly standardized and virus isolation data may vary considerably between laboratories. Sensitivity is highly dependent on the type and quality of specimen, the timing of specimen collection, and storage before arrival in the virus laboratory (Grandien et al., 1995; Morens et al., 1991). The choice of cell types used for EV isolation plays an important role as no single cell line currently in use supports the growth of all known enterovirus serotypes (Rotbart et al., 1985). Hence to ensure maximal isolation efficiency, combinations of cell lines are generally used. The use of multiple cell lines may improve the yield or rapidity with which an EV (Chonmaitree et al.,

1988; Dagan et al., 1986) is isolated at the cost of increasing the labor and resources required. Many EV grow well in primary monkey kidney cells. However the supply of these cells is limited. As many as 25 to 35% of EV serotypes, particularly the coxsackievirus A virus (CAV) group, do not grow at all in cell cultures (Chonmaitree et al., 1982; Lipson et al., 1988; Wildin et al., 1987). Most of them have been propagated in RD cells (Wecker et al., 1977; Schmidt et al., 1975), but practically isolation from clinical specimens is often unsuccessful. The use of suckling mice for isolation of CAV ahs been more successful but because of the practical difficulties this technique is rarely used. The technique is labor intensive and requires a high level of expertise. Other factors affecting the efficiency of isolation are the physiological condition of the cells and whether blind passages are performed. Blind passages are necessary in some cases before a cytopathic effecting cell culture or paralysis in suckling mice becomes apparent. This may be due to the low initial titer of virus or the requirement for adaptation to growth in the isolation systems (Lipson et al., 1988).

3.26 ENTEROVIRUS SEROTYPING:

Typing of viruses is required primarily to provide information on the relationship between viruses, to identify virus types with increased virulence or specific disease attributes, for epidemiological investigations and to allow correlation with viral immunity (Muir et al.,

1998). Following the isolation of an EV, the serotypic identification can be determined by neutralization of infectivity with serotypic specific antisera. Typing by neutralization with reference antisera for all the 66 serotypes individually is clearly impractical. Hence equine type specific hyperimmune sera have been mixed to give intersecting pools containing different combinations of individual antisera (Grandien et al., 1995; Melnick et al., 1996; Schmidt et al., 1961). EV isolates are incubated with each antiserum pool and then reinoculated into susceptible cells. After incubation for several days the neutralization pattern is recorded. Finally the suspected EV type is confirmed by neutralization with the type specific single antiserum. The Lim Benyesh – Melnick (LBM) pool scheme consists of eight pools designated as A to H. containing antisera to 42 different EV types (Lim et al., 1960; Melnick et al., 1973). Antibodies against 19 additional CAV serotypes in seven additional pools designated as J to P can be used if nil result is obtained with A to H pools (Melnick et al., 1977). Alternative intersecting pools have been developed at the National Institute of Public Health and the Environment (RIVM) in the Netherlands, which also allow identification of types 68 to 71 (Kapsenberg et al., 1988). The main disadvantages of using the intersecting pools is that the method is time consuming, labor intensive and costly. Second, the supply of antisera is limited and WHO has advocated a conservative approach to the use of pools, which should not be used to type every clinical isolate (Melnick et al., 1985). Third, the

problem of “untypeable” EV is frequently encountered as several isolates may contain mixture of EVs. Hence to resolve the mixtures the isolates should be purified by three serial terminal dilutions or plaque purifications. All the serotypes cannot be identified by intersecting pools – CAV 3, 11,15,17 and 24 and EV types 68 to 71 cannot be typed using LBM pools. Sometimes EV form aggregates, which can be neutralized only after treatment with sodium deoxycholate or chloroform or by ultrafiltration to dissociate the aggregates (Kapsenberg et al., 1980; Wallis et al., 1967). The isolates may be so called prime strains. These are antigenic variants of recognized serotypes, which are neutralized poorly, or not at all by the antiserum to the homologous prototype strain. However antiserum raised against the prime strain will neutralize both prime and prototypic strains.

3.27 SEROLOGICAL DIAGNOSIS:

Serological diagnosis of EV infections is complicated by the large number of serotypes; the occurrence of anamnestic, heterotypic antibody responses: and the lack of a uniformly cross – reactive EV group antigen. The requirement of appropriately timed paired sera to demonstrate a diagnostically significant rise in antibody titer further limits the utility of EV serologic testing. Detection of IgM in serum may be more sensitive than virus isolation in detecting EV infection (Bell et al., 1986; Davidson

et al., 1995) this provides only circumstantial evidence of an etiological role in concurrent symptoms.

3.28 ANTIGEN DETECTION:

The absence of a widely shared antigen has hampered the development of immunoassays for the EVs much as it has restricted the use of serologic assays (Herrmann et al., 1979; Yolken et al., 1980; Yolken et al., 1981).

3.29 NUCLEIC ACID HYBRIDIZATION:

The earliest efforts at RNA – RNA hybridization for the EVs were designed to explore the genetic similarity among seemingly distantly related serotypes (Young et al., 1973). Early and very limited sequence comparisons confirmed genomic regions of great similarity among certain serotypes, as did heteroduplex-mapping techniques using electron microscopy (Cumakov et al., 1979). Hybridization technology has also been successfully used to detect EV RNA in clinical material from patients with suspected EV infection (Archard et al., 1988a; Archard et al., 1988b; Bowles et al., 1986; Cova et al., 1988)

3.30 cDNA probes:

Early hybridization studies using viral RNA labeled during replication within cells (Young et al., 1973) or cDNA labeled directly during

reverse transcription (Tracy et al., 1981) identified homologies among a number of EVs. With the advent of molecular cloning techniques, segments of several EVs were successfully coned into plasmid vectors, permitting the development of well-defined cDNA probes for the further study of genomic similarities among the serotypes. Probes derived from the 5' noncoding region of the EV genome and others, which included the proteinase, and Polymerase- coding regions in the 3' half of the genome appeared to be the most broadly cross-reactive (Rotbart et al., 1991).

3.31 RNA PROBES:

The commercial availability in 1984 of transcription vectors (Green et al., 1983) made it possible to prepare RNA probes from the cDNA sequences described. There are several theoretical advantages of single stranded RNA probes over cDNA probes. RNA – RNA hybrids have greater affinity than do DNA – RNA hybrids, there are no vector sequences present to cause nonspecific hybridization, and there is no self- annealing among probe strands. A further advantage is the ability to separately produce labeled RNA transcripts of both senses (Rotbart et al., 1991).

3.32 RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS:

The site of cleavage of DNA by a restriction endonuclease is sequence dependent. The presence of mutations at potential cleavage sites in some viral strains results in different patterns of fragments when they are separated in an agarose gel, a phenomenon termed, "Restriction Fragment Length Polymorphism" (RFLP). The technique requires fairly large amount of purified or partially purified viral DNA, a set of restriction enzymes to enzymatically cleave the DNA, the ability to separate the resulting DNA fragments by electrophoresis, and a method of documenting the results. The results are displayed as patterns of bands in an ethidium bromide - stained agarose gels. Viruses with large genomes may have 20 – 50 bands, while viruses with smaller genomes have only 5 – 10 bands. The restriction enzyme used for the digestion is an important factor in the resultant number of bands, and care must be taken that digestion is complete so that partial digestion products do not obscure the final analysis of bands. Restriction endonucleases have recognition sites usually consisting of either four or six bases in a specific palindromic sequence. If a mutation occurs in the DNA genome to either eliminate or create a restriction site, polymorphisms occur in the patterns of bands in the gel. A distinct limitation of this method is that, the presence of mutation cannot be detected unless that mutation happens within the recognition sequence of the restriction endonucleases being used for the

digestion of the DNA. The use of different restriction enzymes will optimize the probability of detecting mutations in a particular genome or portion of a genome. The absence of differing restriction patterns, might be because of testing with too few restriction enzymes (Arens et al., 1999).

3.33 OLIGONUCLEOTIDE FINGERPRINT ANALYSIS:

Oligonucleotide fingerprint analysis, a technically complex method of analyzing randomly distributed non- contiguous segments of an RNA molecule or RNA virus genome, was first described by De Watcher and Fiers (Watcher et al., 1972). The method employs digestion of radioactively labeled genomic RNA with RNase T_1 an endogenous purified from *Aspergillus oryzae* that cleaves single – stranded RNA on the 3’ side of G residues, and two – dimensional electrophoresis. The electrophoresis procedure is performed on 8% polyacrylamide in 6M urea at 4° C in the first direction and then on 22% acrylamide in Tris – borate buffer at room temperature in the second direction after a 90% rotation from the first direction. ^{32}P- labeled RNA oligonucleotides are detected by autoradiography as a fan shaped array of spots and are distributed according to size and composition. The method allows comparison of the larger T_1 – resistant oligonucleotide present in the RNA being tested. This method is considered to be the most sensitive method for analysis of RNA genetic relatedness (Arens et al., 1999).

3.34 RNASE PROTECTION ANALYSIS:

RNase protection analysis was developed by Myers et al., 1985 for the purpose of demonstrating the presence and approximate locations of point mutations in DNA. The technique involves the use of a relatively short, *in vitro* synthesized, radioactive RNA probe that is transcribed from wild type genomic DNA. The technique involves the use of relatively short, in vitro synthesized, radioactive RNA probe that is transcribed from wild type genomic DNA from a standard strain or the type strain of an organism. In this original method, this RNA probe is hybridized to DNA from the test strains prior to the digestion of the heteroduplex with RNase A. The resulting fragments are separated in PAGE containing 8M urea. RNase A cleaves the radioactive RNA probe at positions of mismatch with the DNA. This method detects about half of all single base mutations (Arens et al., 1999).

3.35 SINGLE STRANDED CONFORMATION POLYMORPHISM ANALYSIS:

Single stranded conformation polymorphism analysis is the term applied to the method developed by Orita et al., 1989, with which they demonstrated that single nucleotide substitution was sufficient to cause a mobility shift of a fragment of single stranded DNA in a neutral polyacrylamide gel. Initially the general procedure was to use the RFLP fragments from genomic DNA, denature the fragments by alkali treatment,

and then subject them to electrophoresis in a neutral polyacrylamide gel and compare their mobilities. Modifications have been made to accommodate PCR amplification of a specific region of wild type or mutant genomes prior to denaturation and separation on a neutral gel (Hayashi et al., 1991). In either case if mutations are present in the segment of the mutant genome being tested, that segment will likely run at a different position in the gel than the same segment from the wild type genome. The altered electrophoresis pattern is apparently due to the mutation – altered secondary structure of the restriction fragment or the PCR amplicon (Orita et al., 1989). The seperation of the wild and mutant fragments is dependent on several environmental factors, including the temperature of the gel during electrophoresis, the concentration and composition of the electrophoresis buffer, and the presence of denaturing agents in the gel. The main advantage of this method is that it can "Sample" the genetic make up of several hundred pairs of DNA, whereas RFLP analysis can sample only a few bases (Arens et al., 1999).

3.36 ADENOVIRUS:

Adenovirus is a nonenveloped, double – stranded DNA virus. Viral DNA and associated core proteins are encased in an icosahedral capsid, with 20 triangular faces composed largely of the major capsid protein, hexon. An elongated fibre projects from each of the 12 five fold capsid vertices. At its proximal end, the fibre is bound to a pentameric structure, the penton

base; at its distal end, the fibre forms a globular “knob” domain. In general, the fibre knob functions as the major attachment site for cellular receptors, while the penton base is involved in secondary interactions that are required for virus entry into the cell (Rux et al., 2004; Zhang et al., 2005). There are currently 51 recognised Ad serotypes that have been grouped into six different species, A to F based on their physiochemical, biological and genetic properties (De Jong et al., 1999; Wandell et al., 1984). Serotypes of subgenus A are isolated almost exclusively from the gastrointestinal tract (Schmitz et al., 1983). Adenoviruses of subgroup B such as Ad type 3 and Ad7, and subgroup C are common causes of respiratory tract infections (Sprengel et al., 1994; Wandell et al., 1984). Certain members of the subgroup D Ad 8, Ad19, Ad 37 cause outbreaks of conjunctivitis and rapid identification of these serotypes can help in prevention and control (Elnifro et al., 1999). Subgroup E has one member Ad4, which can cause either respiratory or eye infection, but a genotype variant of Ad4 has been associated with outbreak of conjunctivitis. Infantile gastroenteritis caused by Ad40 and Ad41, which belongs to subgenus F (Adrian et al., 1997). Conventional methods such as isolation in cell culture, direct detection by immunospecific methods, or visualization by electron microscopy, are lacking in rapidity or sensitivity. Identification of Ad subgroups or serotypes can be achieved, with different degrees of efficiency, by serotype specific NT tests (Hierholzer et al., 1995). RE analysis of DNA extracted from infected cells, and more

rarely nowadays, the Hemagglutination inhibition test. Up to thirty days may be required for complete characterization following the initial isolation of Ad in cell culture, which may itself require thirty days or more. In addition, certain Ad such as Ad8, Ad40 and Ad41 are fastidious, with slow and inefficient growth in cell culture (De Jong et al., 1983; Wigand et al., 1987). Consequently, detection by nucleic acid testing such as PCR has been proposed to address these deficits (Echavarria et al., 1999; Echavarria et al., 2001; Walls et al., 2003). Accordingly several PCR based detection methods that target genome regions conserved among adenovirus serotypes and clinical applications have been described by many authors (Avellon et al., 2001; Dalapathy et al., 1998; Echavarria et al., 1998; Echavarria et al., 2000; Echavarria et al., 2003; Hierholzer et al., 1993). PCR based subgenus typing has also been developed and studied by various authors (Kidd et al., 1996; Akerblom et al., 1994; Akerblom et al., 1997; Akalu et al., 1998; Allard et al., 1994).

4. Materials and Methods

4.1 Patients and specimens:

Number of specimens collected for the detection of RV – 335

Types of clinical specimens-

- 184 lens aspirates, 100 peripheral blood samples and 45 urine samples collected from congenital cataract patients within the age group of 6days to one year.
- Six amniotic fluid specimens collected from six fetuses suspected to have congenital infection.

Number of Patients included in the study – 190

Number of isolates obtained from the study – 20

Period of study – June 2003 – May 2006

Number of specimens collected for the detection of HSV – 617

Types of clinical specimens-

- 359 Cerebrospinal fluid specimens, 173 Intraocular specimens, 50 lens aspirates, 11 swabs collected from oral, 9 peripheral blood specimens, 7 swabs taken from genital lesions, 3 swabs from skin blister and 5 miscellaneous specimens consisting of tracheal aspirate and Broncho alveolar lavage (BAL).

Number of Patients included in the study – 609

Number of isolates obtained from the study – NIL

Period of study – June 2003 – March 2006

Number of conjunctival swab (CS) specimens collected for the detection of Ad, CA24v and EV– 157

Number of Patients included in the study – 115 patients with epidemic acute hemorrhagic conjunctivitis

Number of isolates obtained from the study –

Ad: 12 isolates obtained from 157 conjunctival swab specimens

CA 24 v: 50 isolates obtained from 157 conjunctival swab specimens

Period of study – June 2006 – September 2006

Place of study –

The investigations were carried out in L & T Microbiology Research center, Vision Research Foundation, 18, College Road, Chennai – 600 006.

4.2 Basic protocols:

4. 2. 1 Tissue culture cell lines used in the study:

Vero – supplied by National facility for Animal tissue culture (NFATCC), Pune, India

SIRC – kindly provided by Dr. Savithri Sharma, L V Prasad Eye institute, Hyderabad, India

HeLa – obtained from National Centre for Cell Science (NCCS), Pune, India

HEp-2 – obtained from NCCS, Pune, India

4. 2.2 Standard strains:

HSV 1 American Type Culture Collection (ATCC) 733 VR – obtained from Chemicon, USA

HSV 2 SP 753167 – obtained from National Institute of Virology (NIV), Pune, India

Cytomegalovirus AD169 strain – kindly provided by Dr. Sridharan, Christian Medical College, Vellore

VZV – oka vaccine strain; Varilix vaccine, obtained from Smithkline Beecham, Belgium

RV HPV77 – obtained from NIV, Pune

CA24 v strain DN19 obtained from Chemicon, USA

Adenovirus serotype 2 – ATCC VR846 obtained from National Institute of Allergy and Infectious diseases, USA

4.3 Virological techniques used in the study:

4.3.1 Processing of Specimens:

1. Specimens collected from Congenital cataract patients include – Peripheral blood sample, urine sample and lens aspirates.
    a. Peripheral blood samples collected from congenital cataract patients:
    5ml of the peripheral blood was collected and 2ml was transferred to a sterile tube having EDTA (1µg/ml) the remaining 3ml was transferred to a plain tube for separation of serum. The 2ml EDTA tube was allowed to stand at 4ºC for 30 – 45 minutes and the plasma was separated into a sterile 1.5 ml PCR vial. The plasma was further processed for RNA extraction. The 3ml blood sample was allowed to clot and then centrifuged at 3,000 rpm for 10 minutes for the serum separation. The serum sample was used for serological investigations by ELISA technique.
    b. Urine specimens collected from congenital cataract patients:
    The urine specimen was collected aseptically in a sterile container and was centrifuged at 3,000 rpm for 10 minutes. The deposit was used for RNA extraction and stored in - 80º C until inoculated into appropriate cell lines for virus isolation.
    c. Lens aspirates (LA) collected from congenital cataract patients were processed as follows-
    LA was collected in a syringe attached to a vitreous cutter at the beginning of the procedure and transported within 15 minutes to the

microbiology laboratory. The lens aspirate specimen was diluted in 1 ml of Dulbeccos' Minimum Essential Medium (DMEM) with 3% fetal calf serum and stored at -80ºC (International Scientific systems) until inoculated onto cell cultures for isolation of the virus and RNA extraction.

2. Conjunctival swab specimens collected from epidemic acute hemorrhagic conjunctivitis (EAHC) were processed as follows –

   The lower fornix of the conjunctiva was lowered and using a sterile cotton swab the exudate conjunctival secretions was collected in sterile transport medium, which consists of 3% DMEM. The swab was placed in a sterile 1.8 ml cryovial. The swab was cyclomixed and the swab was squeezed onto the inner sides of the cryovial. The transport medium containing the conjunctival specimen was stored at - 80º C until used for Nucleic acid extraction (DNA and RNA) and virus isolation. The pictorial representation of the swab collection is shown in figure1

3. Cerebrospinal fluid (CSF) specimens collected from suspected meningitis and encephalitis patients were processed as follows –

   The specimen was collected in a sterile tube and transported within 2- 3 hours. The specimen was centrifuged at 3,000 rpm for 10 minutes and the deposit was reconstituted in nearly 0.75ml of the supernatant and then stored in - 80º C until used for DNA extraction.

Figure 01

Pictorial representation of conjunctival swab collection

4. Amniotic fluids collected from fetuses suspected to have congenital infection:

   The specimen was collected in sterile tube and then transported within 2 – 3 hours in ice. The specimen was centrifuged at 3,000 rpm for 10 minutes. The deposit was used for RNA extraction and stored in - 80° C until inoculated onto appropriate cell lines for virus isolation.

5. Ocular specimens included in the study were corneal scrapings, Aqueous humor, Vitreous fluid and corneal button.

   A minimal amount of intraocular specimens approximately 50µL of the collected clinical specimen placed in 1ml of Dulbecco's minimum essential medium (DMEM) containing 3% fetal calf serum (FCS) was used for virus isolation with the remaining part processed for DNA extraction. Since the intraocular specimens have a minimal cellular material, they were centrifuged at 10,000 rpm at 4^0C for 15 minutes and the deposit was used for DNA extraction (Priya et al., 2003).

   Corneal scrapings were collected in sterile transport medium and the blade was cyclomixed and the blade was discarded. The specimen was centrifuged and then the deposit was used for DNA extraction. The specimen was stored in -80°C until used for virus isolation (Priya et al., 1999).

6. Miscellaneous specimens consisting of Genital swabs and Oral lesions:

   The specimens were collected using a sterile cotton swab and then placed

in viral transport medium for virus isolation and smears were also made for in direct immnuofluorescence staining.

4.3.2 Indirect Immunofluorescence (IIF) staining technique

Smears were made from a variety of specimens and IIF staining was performed for the detection of RV, HSV and Ad.

4.3.2.1 IIF staining for the detection of RV in culture isolates:

IIF staining was performed from suspected culture harvests of vero and SIRC cell line inoculated with clinical specimens such as lens aspirates, peripheral blood leucocytes and urine samples collected from congenital cataract patients.

- The smears were made from deposit of urine, lens aspirates and leucocytes and air-dried.
- The smears were then fixed in methanol for 10 minutes.
- The smears were stained using 25µl of the primary antiserum i.e. the goat anti- RV antiserum (Chemicon, USA) was used in a dilution of 1:100 for 45 minutes in a moist chamber.
- The smears were then washed with phosphate buffered saline with Tween 20 (PBST) and then air-dried.
- The smears were then stained with secondary antibody i.e. FITC conjugated rabbit anti goat immunoglobulin (Chemicon, USA) was used in a dilution of 1:50 and stained for 45 minutes.
- The smears were washed in PBST and air dried followed by the counter staining with 0.05% Evan's blue for 30

seconds.

- The smears were air dried and mounted with Glycerol and seen under Blue filter of the inverted fluorescent microscope (Nikon, Japan).

4.3.2.2 IIF staining for the detection of HSV in clinical specimens:

IIF staining was performed from corneal scrapings, swabs collected from genital ulcers, swabs collected from oral lesions, and cerebrospinal fluid specimens.

- The smears were made as such from the collected material in case of corneal scrapings, swabs and lens aspirates, whereas in case of cerebrospinal fluid specimens the deposit was used for making smear.
- The smears were then air dried and fixed in methanol for 10 minutes.
- The smears were stained using 25µl of the primary antisera i.e. the rabbit anti- HSV antiserum (DAKO A/S, Denmark) was used in a dilution of 1:25 for 45 minutes in a moist chamber.
- The smears were then washed with phosphate buffered
- saline with Tween 20 (PBST) and then air-dried.
- The smears were then stained with secondary antibody i.e.
- FITC conjugated swine anti - rabbit immunoglobulin (DAKO A/S,
- Denmark) was used in a dilution of 1:25 and stained for 45 minutes.

- ❖ The smears were washed in PBST and air dried followed by the counter staining with 0.05% Evan's blue for 30 seconds.
- ❖ The smears were air dried and mounted with Glycerol and seen under Blue filter of the inverted fluorescent microscope.

4.3.2.3 IIF staining for the detection of Ad in CS specimens:

IIF staining was performed from CS specimens collected from patients suffering from epidemic acute hemorrhagic conjunctivitis.

- ❖ The smears were made as such from the collected material and were air dried and fixed in methanol for 10 minutes.
- ❖ The smears were stained using 25μl of the primary antisera i.e. the rabbit anti- Ad antiserum (NIH, USA) was used in a dilution of 1:25 for 45 minutes in a moist chamber.
- ❖ The smears were then washed with phosphate buffered saline with Tween 20 (PBST) and then air-dried.
- ❖ The smears were then stained with secondary antibody i.e. FITC conjugated swine anti – rabbit immunoglobulin (DAKO A/S, Denmark) was used in a dilution of 1:25 and stained for 45 minutes.
- ❖ The smears were washed in PBST and air-dried followed by the counter staining with 0.05% Evan's blue for 30 seconds.

- The smears were air dried and mounted with Glycerol and seen under Blue filter of the inverted fluorescent microscope.

4.3.3 Virus isolation in cell cultures:

Subculturing of cell line:

- The cell line was examined daily under the inverted phase contrast microscopy (Nikon, Japan) and the bottle showing full sheath of growth was selected for sub culturing purpose.
- The bottle was trypsinized using 0.02% Trypsin - EDTA solution and the cells were resuspended in growth medium (10% Dulbeccos Minimum essential medium).
- The cells were then seeded into respectively labelled 24 - well tissue culture (TC) plate (Axygen scientific, Inc. USA) and incubated at 37° C Co_2 incubator (NuAire, USA) (Schmidt et al., 1989).

Inoculation of clinical specimens from congenital cataract patients:

Number of patients involved in the study: 184

Number of clinical specimens collected: The 329 clinical specimens were catogerized into three groups namely – Group A consisting of 135 specimens collected from 45 patients wherein three specimens of peripheral blood samples and lens aspirate and urine sample was collected; Group B - 110 dual samples collected from 55 patients comprising of peripheral blood sample and lens aspirate specimens; Group C – 84 lens aspirate specimens alone collected from 84 patients

- The 24 - well TC plate was examined under the inverted phase contrast microscope for the monolayer of cells.
- When a healthy monolayer cells were seen, the well in the TC plate was used for the virus isolation purpose.
- 100µl of the clinical specimens namely the lens aspirates, urine samples and peripheral blood leucocytes were inoculated into monolayer of individual wells of SIRC and Vero cells in the TC plate.
- The plates were then placed on the rocking platform to increase the contact penetration of virus to cells, in room temperature for 2 hours, following which 2% DMEM was added and incubated at 37° C Co_2 incubator for 7 days.
- The plates were examined daily for the presence of any cytoplasmic or nuclear changes in the inoculated cells.
- All the plates were inoculated with respective cell and virus controls.

Harvesting of cell cultures:

- After the 7 - day incubation period the plates were frozen in -80° C for 45 minutes following which the plates were rapidly thawed at 37° C.
- The process of freeze thawing were done atleast thrice and the harvests were stored in cryovials at -80° C with appropriate labels.
- All the specimens were passaged atleast thrice before considering

it as Negative for RV and HSV isolation.

Inoculation of conjunctival swab specimens collected from patients suffering from EAHC:

One hundred and fifty seven conjunctival swab specimens collected from 115 patients suffering from epidemic acute hemorrhagic conjunctivitis were included in the study. The patients were requested to come for three follow up visits. During the two follow up visits on days 3, 6 the conjunctival swabs were collected. All the specimens were processed for virus isolation.

- The 96 well TC plate was examined under the inverted phase contrast microscope for the monolayer of cells.
- Only when a healthy monolayer cells were seen, the plate was used for the virus isolation purpose.
- 100µl of the conjunctival swab specimen was inoculated into monolayer of individual wells of HEp – 2, HeLa and Vero cells in the TC plate.
    - The plates were then placed on the rocking platform to increase the contact penetration of virus to cells, in room temperature for 2 hours, following which 2% DMEM was added and incubated at 37° C Co_2 incubator for 4 - 7 days.
    - The plates were examined daily for the presence of any cytoplasmic or nuclear changes in the inoculated cells.
    - All the plates were inoculated with respective cell and virus controls.

Harvesting of cell cultures:

- After the 7 day incubation period the plates were frozen in -80º C for 45 minutes following which the plates were rapidly thawed at 37º C.
- The process of freeze thawing were done atleast thrice and the harvests were stored in cryovials at -80º C with appropriate labels.
- All the specimens were passaged atleast thrice before considering it as Negative for Ad and CA24v isolation.

Inoculation of Amniotic fluid specimen collected from fetuses suspected to have congenital infection:

Six amniotic fluids collected from six fetuses suspected to have fetal viral infection were included in the study.

- The 12 well TC plate was examined under the inverted phase contrast microscope for the monolayer of cells.
- Only when a healthy monolayer cells were seen, the plate was used for the virus isolation purpose.
- 100µl of the clinical specimens namely the Amniotic fluid was inoculated into monolayer of individual wells of SIRC and Vero cells in the TC plate.
  - The plates were then placed on the rocking platform to increase the contact penetration of virus to cells, in room temperature for 2 hours,

following which 2% DMEM was added and incubated at 37º C CO_2 incubator for 7 days.

- The plates were examined daily for the presence of any cytoplasmic or nuclear changes in the inoculated cells.
- All the plates were inoculated with respective cell and virus controls.

Harvesting of cell cultures:

- After the 7 - day incubation period the plates were frozen in -80º C for 45 minutes following which the plates were rapidly thawed at 37º C.
- The process of freeze thawing were done atleast thrice and the harvests were stored in cryovials at -80º C with appropriate labels.
- All the specimens were passaged atleast thrice before considering it as Negative for RV and HSV isolation.

Inoculation of ocular specimens:

- The 24 well TC plate was examined under the inverted phase contrast microscope for the monolayer of cells.
- Only when a healthy monolayer cells were seen, the plate was used for the virus isolation purpose.
- 100µl of the clinical specimens namely the AH, VF, Corneal scrapings was inoculated into monolayer of individual wells of Vero cells in the TC plate.

- ❖ The plates were then kept for rocking in room temperature for 30 minutes, following which 1% DMEM was added and incubated at 37° C Co_2 incubator for 7 days.
- ❖ The plates were examined daily for the presence of any cytoplasmic or nuclear changes in the inoculated cells.
- ❖ All the plates were inoculated with respective cell and virus controls.

Harvesting of cell cultures:

- ❖ After the 7 - day incubation period the plates were frozen in -80° C for 45 minutes following which the plates were rapidly thawed at 37° C.
- ❖ The process of freeze thawing were done atleast thrice and the harvests were stored in cryovials at -80° C with appropriate labels.
- ❖ All the specimens were passaged atleast thrice before considering it as Negative for HSV isolation.

Inoculation of swabs collected from genital and oral ulcerative lesions:

Eighteen clinical specimens (11 swabs collected from oral lesions, 7 genital swabs) collected form patients suspected to have herpes infection were include in the study.

- ❖ The 24 well TC plate was examined under the inverted phase contrast microscope for the monolayer of cells.
- ❖ Only when a healthy monolayer cells were seen, the plate was used for the virus isolation purpose.
- ❖ 100µl of the clinical specimens namely swabs collected from genital

lesion and oral lesion were decontaminated with antibiotic mixture and incubated at 37º C for 30 minutes following which the specimen was centrifuged at 3,000rpm for 10 minutes. The supernatant was inoculated into the individual wells of Vero cells in the TC plate.

- ❖ The plates were then placed on the rocking platform to increase the contact penetration of virus to cells, in room temperature for 2 hours, following which 2% DMEM was added and incubated at 37º C Co_2 incubator for 7 days.
- ❖ The plates were examined daily for the presence of any cytoplasmic or nuclear changes in the inoculated cells.
- ❖ All the plates were inoculated with respective cell and virus controls.

Harvesting of cell cultures:

- ❖ After the 7 - day incubation period the plates were frozen in -80º C for 45 minutes following which the plates were rapidly thawed at 37º C.
- ❖ The process of freeze thawing were done atleast thrice and the harvests were stored in cryovials at -80º C with appropriate labels.
- ❖ All the specimens were passaged atleast thrice before considering it as Negative for HSV isolation.

4.5 Molecular biological techniques used in the study:

RNA extraction:

Three methods of RNA extraction were carried out to determine their efficacy. The three methods used were as follows-

a. RNA extraction using conventional Guanidium Thiocynate (GTC) method
b. RNA extraction using TRIzol reagent
c. RNA extraction using commercially available kits

Precautions that was taken before RNA extraction:

Di- Ethyl Pyrocarbonate (DEPC) Treatment:

The vials & tips that was used for the extraction procedure was made RNAse free by treating them with DEPC, as even a very minute RNAse contamination can also lead to false negative results.

Plastic wares: The plastic wares that was used for RNA extraction was treated overnight with 0.05% Di- ethyl pyrocarbonate (DEPC) followed by autoclaving at 15 lbs for 15 minutes. The plastic wares were then dried in hot air oven before use.

Water: The water that was used for the RNA extraction procedure was also made RNAse free by treating the Milli Q water with 0.1% DEPC overnight. The water was autoclaved at 15lbs for 15 minutes and was used for the preparation of 75% ethanol and reconstitution of the extracted RNA.

4.5.1 Method I - Guanidium Thiocynate Method:

Reagents required:

Denaturing solution – 4M Guanidium Thiocyanate, 25 mM sodium citrate, 0.1M 2-Mercaptoethanol and 0.5% N- Lauryl Sarkosine.

Stock Denaturing solution:

250 gm Guanidium thiocyanate in 293 ml of RNAse free water mixed with 17.6 ml of 0.75M sodium citrate, pH 7.0and 26.4 ml of 10% Sarkosyl at 60- 65 °C with stirring. This stock solution has an expiry of upto three months at RT.

Working Denaturing solution:

0.35 ml of β-mercaptoethanol was added per 50ml stock denaturing solution. This working solution has an expiry of upto a month after preparation when stored at RT.

2M sodium citrate, pH 4.0

Phenol, water saturated

49:1 chloroform/ Isoamyl alcohol

100% Isopropanol

75% Ethanol (prepared with DEPC treated water)

0.5% SDS, DEPC treated

Protocol:

100µl of the clinical specimen was taken and centrifuged at 10,000 rpm for 5 minutes and the supernatant was discarded.

a. To the deposit, 0.5 ml of working denaturing solution was added and the cells were lysed by passing through a pipette 7 – 10 times.

b. To the lysed suspension, 50 µl of 2M sodium acetate was added and mixed well followed by the addition of 0.5 ml of water saturated phenol and 200 µl of 49:1 chloroform/ isoamyl alcohol and mixed well.

c. The solution was incubated for 15 minutes at 0- 4°C and centrifuged at 10,000 rpm for 20 minutes at 4°C.

d. The aqueous layer was transferred into a fresh tube and 0.5 ml of 100% Isopropanol was added and mixed gently and incubated at -20°C for 30 minutes.

e. The solution was centrifuged at 10,000 rpm for 10 minutes at 4°C.

f. The supernatant was discarded and the pellet was treated with 150 µl of denaturing solution, followed by the addition of 150 µl of 100% Isopropanol and incubated at -20°C for 30 minutes.

g. The solution was centrifuged at 10,000 rpm for 10 minutes at 4°C.

h. The supernatant was discarded and the RNA was resuspended in 400 µl of 75% ethanol and vortexed and incubated at RT for 15 minutes.

i. The suspension was centrifuged at 10,000 rpm for 5 minutes at 4°C.

j. The supernatant was discarded, and the pellet was air-dried at RT for 15- 20 minutes.

k. The RNA was resuspended in 50µl of DEPC treated water incubated at 55- 60°C for 15 minutes.

l. The extracted RNA was immediately frozen at -80°C until it was further used for RT-PCR (Chomczynski et al., 1987).

4.5.2 Method II – TRIzol extraction method:

a. 100µl of the clinical specimen was taken for the RNA extraction.
b. To this equal volumes of TRI reagent was added and mixed gently.The mixture was left undisturbed at room temperature for 5 minutes, followed by the addition of 100µl of Chloroform. The mixture was immediately cyclomixed for 15 seconds.
c. The mixture was incubated at room temperature for 15 minutes, followed by centrifuging at 10,000 rpm for 15 minutes at 4º C.
d. The RNA gets eluted in the aqueous layer and hence the upper aqueous layer was taken in a fresh RNAse free 1.5ml vial and treated with 250µl of isopropanol and left undisturbed at room temperature for 10 minutes, followed by centrifugation at10,000 rpm for 15 minutes at 4º C.
e. The supernatant was then decanted and the pellet was washed with 500µl of 75% ethanol prepared with DEPC treated Mili Q water.
f. The pellet was air dried for 10 minutes in a clean laminar flow

hood for the ethanol to evaporate and the pellet was reconstituted with 10μl of DEPC treated water and RNA was dissolved by placing the vial in 56° C waterbath for 15 minutes.

g. The extracted RNA was immediately frozen at -80° C.

4.5.3 Method III – Kit extraction method:

Qiagen kits were used for RNA extraction. The kits used were-

- ✓ QIAmp Viral RNA extraction kit
- ✓ QIAmp Blood RNA extraction kit

QIAmp Viral RNA kit:

The kit reagents were reconstituted before use-

The AVL buffer provided in the kit was mixed with carrier RNA at a concentration of 1μg/ml.

560μl of AVL buffer was used for single extraction.

Wash buffers:

Wash buffers 1 and 2 were reconstituted with 100% absolute ethanol accordingly.

Protocol:

a. To 140μl of clinical specimen added 560μl of AVL buffer and cyclomixed for 15 seconds. The vial was left undisturbed at RT for 10 minutes.

b. 560μl of absolute ethanol was added to the mixture and cyclomixed for 15 seconds at RT.

c. The contents of the vial were transferred to column and centrifuged at 8,000rpm for 1 minute.
d. The filtrate obtained was discarded and the column was washed with 500µl of wash buffer1 at 8,000 rpm for 1 minute.
e. The filtrate was discarded and the column was washed with 500µl of wash buffer 2 at 14,000 rpm for 3 minutes.
f. The filtrate was discarded and the RNA was eluted in 60µl of Elution buffer provided in the kit and stored at -80°C until further use.

QIAmp Blood RNA kit:

The kit reagents were reconstituted before use-

The RPE buffer was reconstituted with 44ml of absolute ethanol to obtain 55ml of wash solution.

10µl of β- mercaptoethanol was added to1ml of RLT buffer just before use.

Protocol:

a. 1ml of the blood sample was mixed with 5ml of Erythrocyte Lysis (EL) buffer and kept on ice for 10 – 15 minutes until all the red blood corpuscles (RBC) were lysed.
b. Care was taken that the blood sample was cyclomixed in between for at least two times to enhance lysis of RBC.
c. The mixture was centrifuged at 500 rpm for 10 minutes at 4°C.

d. The supernatant was discarded and the deposit was lysed by the addition of 2ml of EL buffer and vortexed briefly for 10 seconds to remove the residual RBCs.
e. The tube was centrifuged at 500 rpm for 10 minutes at 4°C.
f. The supernatant was discarded and the leucocyte pellet was lysed by the addition of 600μl of RLT buffer (containing β-mercaptoethanol) and vortexed briefly.
g. The lysed pellet was transferred to QIA shredder column and centrifuged at 14,000 rpm for 2 minutes.
h. The shredder was discarded and the lysate was used for the further extraction.
i. The lysate was mixed with 600μl of 70% ethanol prepared with DEPC treated water and transferred to spin column.
j. The column was centrifuged at 10,000 rpm for 15 seconds.
k. The column was washed with 700μl of RW1 buffer and centrifuged at 10,000 rpm for 15 seconds.
l. The flow through was discarded and the column was washed with 500μl of RPE buffer and centrifuged at 10,000 rpm for 15 seconds.
m. The column was again washed with 500μl of RPE buffer and centrifuged at 14,000 rpm for 3 minutes.
n. The flow through was discarded and the RNA was eluted in 50μl of RNAse free water.

o. The extracted RNA was immediately frozen at -80º C.

4.6 Quantification of RNA:

Reagent: 1mM Disodium Hydrogen phosphate

Preparation of alkaline water:

The DEPC treated water was checked for pH and the pH was adjusted to be > 7.5. The water was used for the quantification of RNA.

Protocol:

The RNA extracted by QIAGEN kit method, TRIzol and by the standard Guanidium thiocyanate method was quantified.

10 µl of the extracted RNA was diluted in 990 µl of alkaline water and the absorbance at 260 nm was read using the spectrophotometer.

4.7 Nested Reverse Transcriptase Polymerase Chain Reaction (nRT-PCR) for the detection of RV:

Number of patients included in the study: 190

Number of clinical specimens tested: 335

The 335 clinical specimens were categorized into four groups namely –

Group A consisting of 135 specimens collected from 45 patients, with congenital cataract, wherein three specimens of peripheral

blood samples and lens aspirate and urine sample was collected; Group B - 110 dual samples collected from 55 congenital cataract patients comprising of peripheral blood sample and lens aspirate specimens; Group C – 84 lens aspirate specimens alone collected from 84 congenital cataract patients; Group D – 6 amniotic fluid samples collected from 6 patients suspected to have congenital infection.

Primers:

The primers were within the E1 open reading frame of RV. The primers sequences used for the nested round of amplification are as shown in table 2.Using 10µl of the extracted RNA the first step of cDNA formation and then PCR for amplification was done using a single step RT-PCR kit procured from Qiagen.

The following cocktail was set up for standardization using the extracted RNA- In brief, the reaction consisted of 400µM of each dNTP, 1X buffer, 0.6µM of each forward and Reverse primer and the enzyme mix. The enzyme mix consists of both the Omniscript and Sensiscript reverse transcriptase and Hot start *Taq* DNA polymerase. These enzymes are recombinant heterodimeric enzymes expressed in *Escherichia coli* and both exhibit higher affinity for RNA, facilitating transcription through secondary structures that inhibit other reverse transcriptases.

Table 2: Primer sequences used in nested Reverse Transcriptase Polymerase chain reaction (nRT-PCR) for the detection of RV

Primer	Primer sequences (5' – 3')	Amplified product
R1	5' CAA CAC GCC GCA CGG ACA AC 3'	185 bp
R2	5' CCA CAA GCC GCG AGC AGT CA 3'	
R3	5' CTC GAG GTC CAG GTC CTG CC 3'	143 bp
R4	5'GAA TGG CGT TGG CAA ACC GG 3'	

Polymerase Chain Reaction:

The enzyme mix facilitates both Reverse transcription and Polymerase chain reaction. The reaction mix was incubated in the thermal cycler as follows: 50°C for 30 minutes for reverse transcription followed by the activation of Hot star *Taq* DNA polymerase enzyme and inactivation of Omniscript and Sensiscript Reverse transcriptases at 95°C for 15 minutes. The PCR amplification was then proceeded for 40 cycles, by denaturing the cDNA template at 94°C, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute. The final extension was carried out at 72°C for 5 minutes. For the second round of the nested amplification, 2µl of the first round product was added to 50µl of the PCR mix consisting of 200µM of each deoxynucleoside triphosphate, 10mM Tris-Cl, 0.6µM of R3 and R4 primers and 2.5U of *Taq* DNA polymerase. The amplification was carried as that of the first round except that only 25 cycles was used.

Specificity of the nRT-PCR for the detection of RV:

The specificity of the primers was determined by performing nRT-PCR using the RNA extracted from the following that commonly cause ocular infections and human DNA from peripheral blood leucocytes from healthy blood donors. The bacterial RNAs tested include *Staphylococcus aureus* (ATCC 25293), *Pseudomonas*

aeuroginosa, Mycobacterium tuberculosis and Chlamydia trachomatis serotype A (ATCC VR 517B). The virus RNAs tested include - Adenovirus, Herpes simplex virus -1 (ATCC 733 VR), Cytomegalovirus (AD169), Varicella zoster virus (Oka Vaccine strain) and ECHO 11 (kindly provided by Dr. Nalini Ramamoorthy, King Institute, Chennai). Fungus RNAs includes *Candida albicans* and *Aspergillus flavus*.

Sensitivity of the nRT-PCR for the detection of RV:

The sensitivity of the primers were determined by diluting the initial standard strain inoculated cell culture harvest 10 fold in DMEM, following which the RNA extraction was performed from each of the 10 log dilutions and nRT-PCR was set up for each.

4.8 Nested Reverse Transcriptase Polymerase Chain Reaction (nRT-PCR) for the detection of Human EVs:

Number of patients included in the study: 115

Number of clinical specimens tested: 157

The 157 clinical specimens were collected from patients suffering from EAHC.

Primers:

The primers were within the 5' non coding region of EV. The primers sequences used for the nested round of amplification are as follows-

S1 5' CAA GCA CTT CTG TTT CCC CGG 3'

S2 5' TCC TCC GGC CCC TGA ATG CG 3'

AS1 5' ATT GTC ACC ATA AGC AGC CA 3'

AS2 5' AAA CAC GGA CAC CCA AA GTA 3'

Using 10µl of the extracted RNA the first step of cDNA formation and then PCR for amplification was done using a single step RT-PCR kit procured from Qiagen.

The following cocktail was set up for standardization using the extracted RNA- In brief, the reaction consisted of 400µM of each dNTP, 1X buffer, 0.6µM of each forward and Reverse primer and the enzyme mix. The enzyme mix consists of both the Omniscript and Sensiscript reverse transcriptase and Hot start *Taq* DNA polymerase. These enzymes are recombinant heterodimeric enzymes expressed in *Escherichia coli* and both exhibit higher affinity for RNA, facilitating transcription through secondary structures that inhibit other reverse transcriptases.

Polymerase Chain Reaction:

The enzyme mix facilitates both Reverse transcription and Polymerase chain reaction. The reaction mix was incubated in the thermal cycler as follows: 50°C for 30 minutes for reverse

transcription followed by the activation of Hot star *Taq* DNA polymerase enzyme and inactivation of Omniscript and Sensiscript Reverse transcriptases at 95°C for 15 minutes. The PCR amplification was then proceeded for 40 cycles, by denaturing the cDNA template at 94°C, annealing at 62°C for 30 seconds and extension at 72°C for 1 minute. The final extension was carried out at 72°C for 5 minutes. For the second round of the nested amplification, 2µl of the first round product was added to 50µl of the PCR mix consisting of 200µM of each deoxynucleoside triphosphate, 10mM Tris-Cl, 0.6µM of R3 and R4 primers and 2.5U of *Taq* DNA polymerase. The PCR amplification was carried out for 25 cycles, denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 1 minute.

Specificity of the nRT-PCR for the detection of Human EVs:

The specificity of the primers was determined by performing nRT-PCR using the RNA extracted from the following that commonly cause ocular infections and human DNA from peripheral blood leucocytes from healthy blood donors. The bacterial RNA tested include *Chlamydia trachomatis* serotype A (ATCC VR 517B). The virus RNAs tested include - Adenovirus, Herpes simplex virus -1 (ATCC 733 VR), poliovirus –1, enterovirus 70 and ECHO 11

(kindly provided by Dr. Nalini Ramamoorthy, King Institute, Chennai).

Sensitivity of the nRT-PCR for the detection of Human EVs:

The sensitivity of the primers were determined by diluting the initial standard strain inoculated cell culture harvest 10 fold in DMEM, following which the RNA extraction was performed from each of the 10 log dilutions and nRT-PCR was set up for each.

4.9 uniplex Reverse Transcriptase Polymerase Chain Reaction (uRT-PCR) for the detection of CA24v:

A total of 157 conjunctival swab specimens collected from 115 patients suffering from EAHC were tested for the presence of CA24v.

Primers:

The primers were within the VP1 region of CA24v. The primers sequences used for the uRT-PCR are shown in table 3.

Using 10µl of the extracted RNA the first step of cDNA formation and then PCR for amplification was done using a single step RT-PCR kit procured from Qiagen.

The following cocktail was set up for standardization using the extracted RNA- In brief, the reaction consisted of 400µM of each dNTP, 1X buffer, 0.8µM of each forward and Reverse primer and the enzyme mix. The enzyme mix consists of both the Omniscript

Table 3: Primer sequences used in uniplex Reverse Transcriptase Polymerase chain reaction (uRT-PCR) for the detection of CA24v

Primer	Primer sequences (5' – 3')	Amplified product
F1	5' GCACAAGGCATTGAGGAGACCATT 3'	171 bp
R1	5' TGCCTGGCCIGATACICCAGTCTC 3'	

and Sensiscript reverse transcriptase and Hot start *Taq* DNA polymerase. These enzymes are recombinant heterodimeric enzymes expressed in *Escherichia coli* and both exhibit higher affinity for RNA, facilitating transcription through secondary structures that inhibit other reverse transcriptases. The cocktail was placed in a thermal cycler (Eppendorf) and was run in the following profile- 50°C for 30 minutes for Reverse transcription to proceed 95°C for 15 minutes for Hot start *Taq* DNA polymerase activation & inactivation of Reverse transcriptases.

Thermal Profile:

The thermal profile consists of 40 cycles of -

Denaturation at 94°C for 30 seconds, annealing at 68.1°C for 30 seconds and extension at 72°C for 1 minute followed by a single cycle of final extension at 72°C for 5 minutes.

Specificity of the uRT-PCR for the detection of CA24v:

The specificity of the primers was determined by performing uRT-PCR using the RNA extracted from the following organisms. The bacterial RNA tested include *Chlamydia trachomatis* serotype A (ATCC VR 517B). The virus RNAs tested include - Adenovirus, Herpes simplex virus -1 (ATCC 733 VR), poliovirus –1, enterovirus 70 and ECHO 11 (kindly provided by Dr. Nalini Ramamoorthy, King Institute, Chennai).

Sensitivity of the uRT-PCR for the detection of CA24v:

The sensitivity of the primers were determined by diluting the initial standard strain inoculated cell culture harvest 10 fold in DMEM, following which the RNA extraction was performed from each of the 10 log dilutions and uRT-PCR was set up for each.

4.10 DNA extraction:

DNA extraction from the clinical specimens was done using the clinical genomic DNA mini prep kit, Biogene Inc., CA, USA as per the manufacturer's instructions.

Protocol:

a. 200µl of Digestion buffer was mixed with 100µl of clinical specimen, followed by the addition of 3µl of proteinase K (used at a concentration of 1mg/150µl). The sample was cyclomixed and incubated at 55°C for 5 minutes.

b. To the lysed sample, 200µl of absolute ethanol was added and mixed well. The whole mixture was then transferred to the spin column and centrifuged at 10,000 rpm for 1 minute.

c. The flow through was discarded and the column was washed twice with 500µl of wash buffer and centrifuged at 10,000 rpm for 1 minute.

d. Additional centrifugation was done at 12,000 rpm for 1 minute to remove the residual wash solution.

e. The column was then placed into a clean 1.5ml microfuge tube and the DNA was eluted in 100µl of elution buffer at 50ºC for 2 minutes.
f. The column was spun at 10,000rpm for 1 minute to elute the DNA from the column.

4.10.1 uniplex Polymerase Chain Reaction (uPCR) for the detection of HSV:

21 intraocular fluids collected from 19 patients of which 16 with acute retinal necrosis (ARN), 2 with viral retinitis, and one each of progressive outer retinal necrosis (PORN), CMV retinitis and optic neuritis were also tested for the presence of HSV DNA.

Primers:

Primers for the uniplex PCR for the detection of HSV were targeted against the DNA Polymerase gene of HSV. The primer sequences used are as shown in table 4.

PCR:

This uPCR has been already standardized in our laboratory and this was applied for the evaluation against the other set of primers that was targeted against the Glycoprotein D (gD) gene.

The thermal profile consisted of initial denaturation at 94ºC for 2 minutes, followed by 35 cycles of denaturation, annealing and extension at 94ºC for 45 seconds, 64ºC for 45 seconds and 72ºC for 45

Table 4: Primer sequences used in uniplex Polymerase Chain Reaction (uPCR) targeting DNA Polymerase gene of HSV

Primer	Primer sequences (5' – 3')	Amplified product
F1	5' ATC AAC TTC GAC TGG CCC TT 3'	179 bp
R1	5' CCG TAC ATG TCG ATG TTC AC 3'	

seconds respectively. The sensitivity and specificity of the PCR has been determined already.

4.10.2 semi Nested Polymerase Chain Reaction (snPCR) for the detection of HSV:

A total of 546 clinical specimens (359 Cerebrospinal fluid specimens, 152 ocular specimens, 11 swabs collected from oral lesions, 9 peripheral blood specimens, 7 genital swabs, 3 swabs collected from skin blisters and five miscellaneous specimens consisting three of BAL specimens and one each of tracheal aspirate and LA) collected from 540 patients were tested for the presence of HSV genome.

21 intraocular fluids collected from 19 patients of which 16 with acute retinal necrosis (ARN), 2 with viral retinitis, and one each of progressive outer retinal necrosis (PORN), CMV retinitis and optic neuritis were also tested for the presence of HSV DNA.

50 lens aspirates collected from 50 congenital cataract patients were tested for the presence of HSV DNA.

Primers:

The primers sequences used in the PCR flank the Glycoprotein D gene of HSV genome. The primers sequences used for the semi nested round of amplification are as shown in table 5.

Table 5: Primer sequences used in the seminested Polymerase chain reaction (snPCR) for differentiation of HSV 1 and 2 serotypes

Virus	Primer orientation	Primer sequences (5’-3’)
HSV 1	Outer & Inner sense	5’ CGAAGACGTCCGGAAACAAC 3’
	∗ Outer antisense	5’ CGGTGCTCCAGGATAAA 3’
	∗ Inner antisense	5’ TCTCCGTCCAGTCGTTTATCTTC 3’
HSV 2	Outer & Inner sense	5’ GGACGAGGCCCGAAAGCACA 3’
	∗ Outer antisense	5’ CGGTGCTCCAGGATAAA 3’
	∗ Inner antisense	5’ TCTCCGTCCAGTCGTTTATCTTC 3’

∗ The Outer and the Inner antisense primer sequences are common for both HSV 1 and 2 serotypes.

The following cocktail was set up for standardization using the extracted DNA- The snPCR was performed in a 50µl reaction volume containing 1X PCR buffer (10mM Tris with 15mM $MgCl_2$), 200µm of each dNTPs, 2.5 units of *Taq* DNA polymerase, 1µM each primer (custom synthesized by Bangalore Genei Pvt. Ltd).

Thermal Profile:

Thermal profile for the first round was for 35 cycles with each cycle consisting of three steps denaturation at 94°C / 40 secs, annealing at 50°C / 40 secs and extension at 72°C / 40 secs in the thermal cycler (PE Applied Biosystems 2700, USA). The second round of amplification using the seminested primers was done for 20 cycles of incubation at 94°C, 65°C and 72°C for 40 seconds each

Specificity of the snPCR for the detection of HSV:

The specificity of the standardized PCR was verified by testing the PCR against the several DNA samples extracted from bacteria – *Staphlycoccus aureus* (ATCC 25293), *Chlamydia trachomatis* serotype A (ATCC VR 517B), *Mycobacterium tuberculosis* (H37Rv) and laboratory strains of *Propionibacterium acnes*. Fungal DNA tested included - *Candida albicans*, *Aspergillus flavus*, *Aspergillus niger*. The cross reactivity between the herpes group of viruses were also determined by testing the primers against Varicella zoster virus DNA (Oka vaccine strain), Cytomegalovirus DNA (AD169).

Validation of the type specific PCR with the isolates:

DNAs of 10 random clinical isolates and the ATCC standard strains of HSV 1 and 2 were extracted and snPCR was performed. The results were analyzed. These clinical isolates were earlier sero-typed by us using the Neutralization test, PCR based Restriction Fragment Length Polymorphism (PCR-RFLP) and DNA sequencing.

Sensitivity of the snPCR for the detection of HSV:

The sensitivity of the snPCR was tested by serial ten fold dilutions of the extracted positive control DNA of both HSV -1 and 2 in sterile Milli Q water. snPCR was performed on the diluted samples and the sensitivity was determined.

4.11 PCR based Restriction Fragment Length Polymorphism (PCR-RFLP):

This was used for rapid serotype identification of HSV using the PCR amplified products of gD gene of HSV. Although the primers can specifically serotype HSV, PCR-RFLP was used for further confirmation especially with HSV positive samples.

The restriction enzymes were chosen based on the sequences in the amplified region and the restriction enzymes were verified using the WEBCUTTER software program.

The enzymes chosen for the Serotyping study include – *Hinf I* and *Hae III*

The restriction sites for the enzymes are shown in the figures 2 (a &b)

and 3 (a &b).

Properties of *Hinf I*

Recognition sequence: GANTC

Cleavage site:

5’----G ^ ANTC----3’

Reaction Buffer:

Requires buffer C for its activity containing 10mM TrisHcl pH 7.8, 50mM Nacl, 10mM $Mgcl_2$, 1mM dithiothretiol. Does not require Nuclease free BSA for its activity.

Temperature and time of incubation:

Optimal activity of the enzyme is at 37° C for one to two hours. The enzyme cleaves single stranded DNA with 10% efficiency as compared to double stranded DNA.

Properties of *Hae III*

Recognition sequence: GGCC

Cleavage site:

5’----GG ^ CC ----3’

Figure 2a:Glycoprotein D gene of Herpes simplex virus – 1 with Restriction sites for *Hinf -I*

CGA AGACGTCCGG AAACAACCCT ACAACCTGAC CATCGCTTGG
TTTCGGATGG GAGGCAACTG TGCTATCCCC ATCACGGTCA
TGGAGTACAC CGAATGCTCC TACAACAAGT CTCTGGGGGC
CTGTCCCATC CGAACGCAGC CCCGCTGGAA CTACTATGAC
AGCTTCAGCG CCGTCAGCGA GGATAACCTG GGGTTCCTGA
TGCACGCCCC CGCGTTTGAG ACCGCCGGCA CGTACCTGCG
GCTCGTGAAG ATAAACGACT GGACGGAGA

Hinf I does not have a restriction site for this sequence.

Figure 2b:Glycoprotein D gene of Herpes simplex virus – 2 with Restriction sites for *Hinf -I*

G GACGAGGCCC GAAAGCACAC GTACAACCTG ACCATCGCCT
GGTATCGCAT GGGAGACAAT TGCGCTATCC CCATCACGGT
TATGGAATAC ACCGAGTGCC CCTACAACAA GTCGTTGGGG
GTCTGCCCCA TCCGAACGCA GCCCCGCTGG AGCTACTATG
ACAGCTTTAG CGCCGTCAGC GAGGATAACC TGGG↓ATTCCT
GATGCACGCC CCCGCCTTCG AGACCGCGGG TACGTACCTG
CGGCTAGTGA AGATAAACGA CTGGACGGAG A

The RE enzyme cleaves at 195th position yielding 195bp and 77bp.

Reaction Buffer:

Requires buffer C for its activity containing 10mM TrisHcl pH 7.8, 50mM Nacl, 10mM $Mgcl_2$, 1mM dithiothretiol.

Does not require Nuclease free BSA for its activity.

Temperature and time of incubation:

Optimal activity of the enzyme is at 37° C for one to two hours. The enzyme cleaves single stranded DNA with 10% efficiency as compared to double stranded DNA.

Hinf I:

For restriction enzyme digestion, a 25µl reaction was set up with 2.5µl of buffer C, 3µl of enzyme (30units), 5µl of DNA was added and final volume was made up with Millipore water. The reaction mixture was mixed gently and incubated at 37° C for 2 hours in a thermal cycler.

Hae III:

For restriction enzyme digestion, a 25µl reaction was set up with 2.5µl of buffer C, 3µl of enzyme (30units), 5µl of DNA was added and final volume was made up with Millipore water. The reaction mixture was mixed gently and incubated at 37° C for 2 hours in a thermal cycler.

Figure 3a:Glycoprotein D gene of Herpes simplex virus – 1 with Restriction sites for *Hae - III*

CGA AGACGTCCGG AAACAACCCT ACAACCTGAC

CATCGCTTGG TTTCGGATGG GAGGCAACTG TGCTATCCCC

ATCACGGTCA TGGAGTACAC CGAATGCTCC TACAACAAGT

CTCTGGG**GG↓C C**TGTCCCATC CGAACGCAGC CCCGCTGGAA

CTACTATGAC AGCTTCAGCG CCGTCAGCGA GGATAACCTG

GGGTTCCTGA TGCACGCCCC CGCGTTTGAG ACCGCCGGCA

CGTACCTGCG GCTCGTGAAG ATAAACGACT GGACGGAGA

The RE enzyme cleaves at 122nd position yielding 150bp and 122bp.

Figure 3b:Glycoprotein D gene of Herpes simplex virus – 2 with Restriction sites for *Hae - III*

G GACGA**GG↓CC**C GAAAGCACAC GTACAACCTG

ACCATCGCCT GGTATCGCAT GGGAGACAAT TGCGCTATCC

CCATCACGGT TATGGAATAC ACCGAGTGCC CCTACAACAA

GTCGTTGGGG GTCTGCCCCA TCCGAACGCA GCCCCGCTGG

AGCTACTATG ACAGCTTTAG CGCCGTCAGC GAGGATAACC

TGGGATTCCT GATGCACGCC CCCGCCTTCG AGACCGCGGG

TACGTACCTG CGGCTAGTGA AGATAAACGA CTGGACGGAG A

The RE enzyme cleaves at 8th position yielding 264bp and 8bp.

Storage of restriction enzymes:

All restriction enzymes are stored at -20° C in non frost- free freezer. The enzyme is just removed prior to use and kept on ice. The enzyme must be kept immediately in freezer as soon as possible. It is best to store the enzymes as supplied in the concentrated form. If the enzyme must be diluted recommended enzyme storage buffer should be used.

Glycerol is added to the restriction enzymes to prevent freezing at -20° C as repeated freeze thawing can reduce their activity. Some restriction enzymes show reduced specificity or increased star activity when glycerol concentration in the final reaction is higher than 5% though many have normal specificity or glycerol concentration as high as 10% (Ingawa et al., 1996).

4.12 Real time Polymerase chain reaction for quantitation of HSV particles:

Total of 50 lens aspirate specimens collected from 50 congenital cataract patients were included in the study. These specimens were qualitatively tested for the presence of HSV DNA. Nine specimens were positive for HSV and these were further quantitated using Real time PCR.

The primers and probes used for the real time PCR was within the DNA Polymerase gene of HSV. The primers can amplify both HSV 1 and 2 serotypes. The probe sequences used in the kit are as follows – 5' CTT ACC CCC GTA GAT GAC GCC 3'. 50µl reaction was set up as per the

manufacturers' instructions. Each run was carried out with the five standards provided in the kit for quantitation purpose. The thermal profile consists of initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation, annealing and extension at 95°C for 15 seconds, 55°C for 20 seconds and 72°C for 15 seconds respectively. The dyes used were FAM and JOE.

By comparing with the standards the quantitation data was obtained.

4.13 semi nested Polymerase Chain Reaction (snPCR) for the detection of VZV:

50 lens aspirate specimens collected from 50 congenital cataract patients were tested for the presence of VZV DNA.

21 intraocular fluids collected from 19 patients of which 16 with acute retinal necrosis (ARN), 2 with viral retinitis, and one each of progressive outer retinal necrosis (PORN), CMV retinitis and optic neuritis were also tested for the presence of VZV DNA.

Primers:

The semi-nested sets of primers are targeted against the immediate early 63 gene of VZV. The primer sequences used are as follows –

VZV1: 5' CAGTTCATCCGCAGACTCCAACGC 3'

VZV2: 5' TACGGACATGAACTTTATCGTACC 3'

VZV3: 5' CTTTATCGTACCTTTGAGTC 3'

PCR:

This snPCR has been already standardized in our laboratory and this was applied onto clinical specimens for the detection of VZV. The thermal profile consisted of initial denaturation at 94°C for 2 minutes for 1 cycle followed by 35 cycles for the first round of amplification and 15 cycles for the second round, each consisting of denaturation at 94°C for 45 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 45 seconds respectively. The sensitivity and specificity of the PCR has been determined already.

4.14 Nested PCR for the detection of CMV:

50 lens aspirate specimens collected from 50 congenital cataract patients were tested for the presence of CMV DNA.

21 intraocular fluids collected from 19 patients of which 16 with acute retinal necrosis (ARN), 2 with viral retinitis, and one each of progressive outer retinal necrosis (PORN), CMV retinitis and optic neuritis were also tested for the presence of CMV DNA.

Primers:

The nested sets of primers are targeted against the morphological transforming region II (mtrII) of CMV. The primer sequences used are as follows –

F1 5' CTG TCG GTG ATG GTC TCT TC 3'

R1 5' CCC GAC ACG CGG AAA AGA AA 3'

F2 5' TCT CTG GTC CTG ATC GTC TT 3'

R2 5' GTG ACC TAC CAA CGT AGG TT 3'

PCR:

This nPCR has been already standardized in our laboratory and this was applied onto clinical specimens for the detection of CMV. The thermal profile consisted of 35 cycles of denaturation at 94°C for 45 seconds, annealing at 55°C for 45 seconds and extension at 72°C for 45 seconds. The second round was repeated in the same thermal profile except that 25 cycles of amplification was done. The sensitivity and specificity of the PCR has been determined already.

4.15 Nested Polymerase Chain Reaction (nPCR) for the detection of Ad:

157 clinical specimens collected from 115 patients suffering from EAHC were included in the study.

Primers:

Primers for the nested round of amplification for the detection of Ad were targeted against the hexon gene of Ad. The primer sequences used are as shown in table 6.

PCR:

This nPCR has been already standardized in our laboratory and this was applied onto the conjunctival swab specimens collected from patients suffering with EAHC. The thermal profile consisted of 36 cycles of denaturation, annealing and extension at 94°C for 1 minute, 50°C for 1

minute and 72°C for 2 minutes respectively. The final extension was for 72°C for 7 minutes. The sensitivity and specificity of the PCR has been determined already.

Table 6: Primer sequences used in nested Polymerase chain reaction (nPCR) for the detection of Ad

Primer	Primer sequences (5' – 3')	Amplified product
F1	5' GCC ACC TTC TTC CCC ATG GC 3'	1006 bp
R1	5' GTA GCG TTG CCG GCC GAG AA 3'	
F2	5' TTC CCC ATG GCN CAC AAC AC 3'	960 bp
R2	5' GCC TCG ATG ACG CCG CGG TG 3'	

DNA sequencing:

The 50μl amplified products of the clinical samples were run on a 2% agarose gel and electrophoresed. The respective amplified product was visualized in the UV transilluminator and the band was cut using a sterile blade and transferred into a 1.5ml microfuge tube. The DNA was eluted from the agarose gel using the Mini Gel elution kit from Eppendorf, USA. The DNA was eluted in a total of 30μl elution volume and cycle sequencing was set up using 2μl of the eluted product.

Elution of DNA from Agarose gel:

The protocol was followed as per the manufacturers instructions, which basically consisted of the following steps-

a. The DNA band, which was excised, was weighed and to that 3 volumes of binding buffer was added for every 1 volume of gel slice.

b. The gel with the binding buffer was incubated at 50°C for 10 minutes.

c. Precaution was taken that every two minutes the mixture was cyclomixed to enhance solubilization of the gel.

d. After ten minutes the mixture was examined for any agarose particles and then to it 1 volume of isopropanol was added and mixed by repeated pipetting.

e. The mixture was transferred to the spin column provided in the kit and centrifuged at 15,000rpm for 1 minute.

f. The flow through was discarded and the column was washed with 750μl of freshly prepared wash buffer consisting of 1 part of wash concentrate with 4 parts of absolute ethanol.
g. The flow through was discarded and the column was spun for additional 1 minute at 15,000rpm to remove the residual was buffer.
h. The DNA was eluted in a fresh tube by the addition of 30μl of elution buffer provided in the kit and centrifuging at 15,000rpm for 1 minute.

CYCLE SEQUENCING:

Cycle sequencing was performed using the following reagents -

Ready Reaction Mix (RR mix) – varies from0.5 - 3μl (depends on the bp size)

Buffer - 3μl

Forward primer of second round - 2μl

Milli Q water - 2μl

Respective amplified product - 2μl

NOTE: Depending on the amount of RR mix added the other reagents were adjusted accordingly to a final volume of 10μl.

The reagents were mixed and run in the following universal thermal profile- initial denaturation at 96º C for 1 minute, followed by 25 cycles of denaturation, annealing and extension at 96º C for 10 seconds, 50º C for 5 seconds and 60º C for 4 minutes respectively.

PURIFICATION OF THE CYCLE SEQUENCED PRODUCT:

a. To the 10µl of the cycle sequenced product, 10µl of sterile Millipore water was added and the contents were transferred to a 0.5 ml microfuge tube. 2µl of 125mM EDTA and 2µl of 3M sodium acetate was added followed by the addition of 50µl of chilled absolute ethanol.
b. The 0.5ml microfuge tube was cyclomixed after addition of the reagents and incubated at room temperature for 15 minutes.
c. The tube was centrifuged at 12,000 rpm for 25 minutes.
d. The supernatant was discarded and the pellet was washed with 70% ethanol twice at 10,000 rpm for 10 minutes.
e. The vial was dried at 37° C overnight, following which the DNA was denatured by the addition of 13µl of Hi dye formamide and heat denatured at 90° C for 3 minutes and loaded onto the plates for sequencing to proceed.

4.16 AGAROSE GEL ELECTROPHORESIS:

Reagents required:

Stock 10X Tris Boricacid EDTA buffer (10XTBE):

Prepared by adding 54.1gm of Tris base to 200ml of Millipore water and dissolved. Then 3.65gms of EDTA was added and dissolved by further addition of 100ml of Millipore water. To 300ml of the mixture, 27.8gms of boric acid was added and remaining 200ml of Millipore water. The pH was adjusted to 8.0.

Working 1X TBE buffer:

The stock buffer was diluted 1:10 with Millipore water and used for preparing agarose gel.

Ethidium bromide:

Stock consists of 2mg/ml of Millipore water; the working concentration used is 0.5µg/ml.

Bromophenol Blue (BPB):

Prepared by adding equal volumes of 0.1% BPB in 1XTBE and 40% sucrose solution in water.

Detection of amplified product

a. 2 % agarose gel was prepared in 1X TBE buffer by melting the agarose in microwave oven.

b. Then 2 mg/ml of Ethidium Bromide was added to the melted agarose in a final concentration of 0.5mg/ ml and mixed well.

c. This was cast onto a gel trough already sealed on both the sides with cellophane tape and fixed with gel combs of the desired well size.

d. The gel was allowed to solidify in dark to reduce the photo activation of Ethidium Bromide.

e. Cellophane tape and combs were removed and the trough was placed at the cathode end of the buffer tank containing 1 X TBE.

f. Care should be taken that the gel is completely immersed in the buffer.

g. To 2 μl of bromophenol blue, 10 μl of the amplified product was added mixed well and loaded onto the corresponding wells.

h. The amplified products were loaded in order and results were read by comparing the amplified product size with that of the molecular weight marker.

i. The molecular weight marker loaded contains 1 μl of molecular weight marker, 7μl of 1 X TBE and 2 μl of bromophenol blue.

j. The electrophoresis was run at 100 volts and after 30 – 45 minutes the gel was seen under the transilluminator.

5. RESULTS

5.1 Results of comparison of RNA extraction methods:

Three methods of RNA extraction were standardized and used namely – Guanidium Thiocyanate method, TRIzol reagent and kit method of RNA extraction. The quantitation of RNA thus obtained from each of the three methods was spectrophotometrically estimated at 260nm and optical density (OD) readings were recorded. RNA extracted from eighty-six samples (50 of lens aspirates, 22 peripheral blood leucocytes, 4 aminotic fluid samples, 10 urine samples) were used for evaluation. An OD corresponds to the amount of nucleic acid in µg in a one ml volume using a 1cm path length quartz cuvette that resulted in an OD260 reading of 1. For RNA, OD260 1 corresponds to 40 mg / ml. The purity of the extracted RNA by the three methods was verified by recording the OD readings at 280nm. The ratio of readings taken at 260nm and 280nm wavelengths indicates the purity of the nucleic acid. For pure RNA, the ratio should be greater than or equal to 2.0. The guanidium thiocyanate method yielded RNA two fold higher than the other two methods. The kit method was found to be efficient for cell free specimens like lens aspirates, amniotic fluids and some urine samples. Except for 2 urine samples and 1 peripheral blood leucocyte, all the RNA obtained were pure by all the three methods.

5.2 Standardization of nRT-PCR for the detection of RV genome in clinical specimens:

The nRT-PCR was standardized using the standard strain of RV.

Specificity: The optimized nRT-PCR for E1 gene was specific for RV genome since there was no amplification of RNAs from infective agents other than RV and human RNA included in the reactions as shown in figure 4.

Sensitivity: The sensitivity of the primers was determined using the RNA extracted from the each of the 10 log dilutions. The standardized nRT-PCR was set up for each of the dilutions and the detection limit was found to be 10 femtograms (fg) equivalent to 10 viral particles. The agarose gel photograph showing the sensitivity of the primers is depicted in figure 5.

Results of application of standardized nRT-PCR onto clinical specimens:

A total of 335 clinical specimens collected from 190 patients, which included 189 patients clinically diagnosed to have congenital cataract and one patient with developmental cataract, were analyzed for the presence of RV genome. The 335 clinical specimens were catogerized into four groups namely –

Figure 04

Agarose gel electrophoretogram showing the specificity of the E1 gene primers used in the nRT-PCR for the detection of RV genome

Lanes NC, NC1 – Negative controls

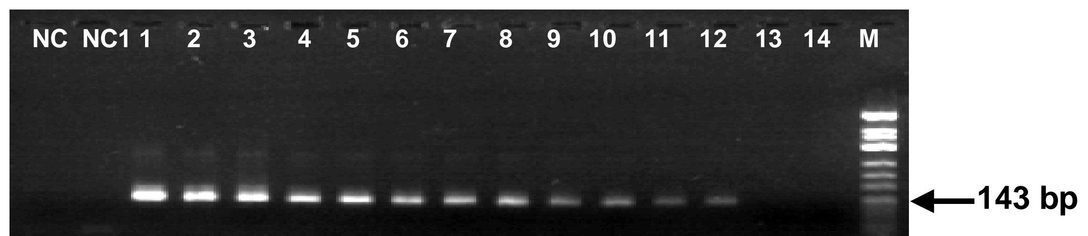
Lanes 1 – 10 – RNA from infective organisms

Lane P – Rubella virus RNA

Lane M - DNA ladder (ϕ X 174 DNA / *Hinf* I digest)

Figure 05

Agarose gel electrophoretogram showing the sensitivity of the E1 gene primers used in the nRT-PCR for the detection of RV genome



Lanes NC, NC1 - Negative controls

Lane 1 – Neat Rubella virus RNA

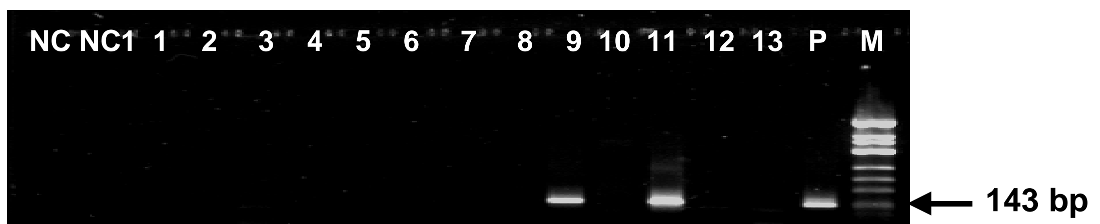
Lanes 2 – 14 Log $_{10}$ dilutions of Rubella virus RNA

Group A consisting of 135 specimens collected from 45 patients wherein three specimens of peripheral blood samples and lens aspirate and urine were collected; Group B - 110 dual samples collected from 55 patients comprising of peripheral blood sample and lens aspirate specimens; Group C – 84 lens aspirate specimens alone collected from 84 patients; Group D – 6 amniotic fluid samples collected from 6 patients.

In Group A, two of the 45 patients showed the presence of RV genome in all their three specimens viz. lens aspirates, peripheral blood leucocytes and urine specimens. RV genome was detected in lens aspirate and peripheral blood leucocyte specimens in additional four patients. Four of the 45 patients showed the presence of RV genome in their lens aspirates alone. Hence a total of 18 specimens were positive for RV genome in group A. In group B, three patients showed the presence of RV genome in dual specimens collected viz., peripheral blood leucocyte specimen and lens aspirates. Six lens aspirate specimens alone were positive for RV genome in this group. Ten out of the 84 patients indicated the presence of RV genome in their lens aspirates in group C. Group D which comprises of the amniotic fluid samples collected from pregnant mothers suspected to have congenital infection, two samples out of six showed the presence of RV genome as shown in figure 6. The summary of the results of each group is shown in table 7.

Figure 06

Agarose gel electrophoretogram showing the application of nRT-PCR onto clinical specimens for the detection of RV genome



Lane NC, NC1 - Negative controls

Lanes 1 – 13 - Lens aspirate specimens 1 - 13

Lanes 9, 11 – Lens aspirate POSITIVE specimens

Lane P – HPV77 standard strain - Positive control

Lane M - φ X 174 *Hinf I* digest Molecular weight marker

Table 7: Results of application of nRT-PCR onto clinical specimens for the detection of RV genome

S. No	Group	Clinical specimen	RV genome detected
1	A• (n=45)	Lens aspirates	10
		Peripheral blood leucocytes	06
		Urine	02
2	B♦ (n=55)	Lens aspirates	09
		Peripheral blood leucocytes	03
3	C (n=84)	Lens aspirates	10
4	D (n=6)	Amniotic fluid	02

Note: 'n' indicates the number of patients

- In two patients, all the three specimens were positive for RV.
- In four patients, Lens aspirate and peripheral blood leucocytes were positive for RV
- In four patients lens aspirates alone were positive.

♦ In three patients, both lens aspirates and peripheral blood leucocytes were positive for RV.

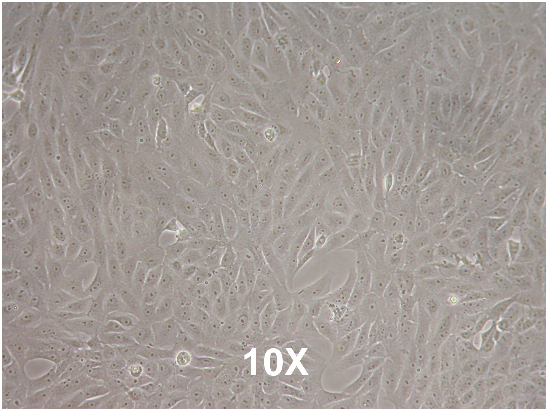
♦ In six patients, lens aspirates alone were positive for RV.

5.3 Results of conventional method:

The conventional method of RV detection was done using virus isolation by cell cultures. Since this virus does not show significant CPE, the replication of RV in cell cultures was confirmed by two techniques namely – IIF technique and by application of the standardized nRT-PCR for the detection of RV genome as per the recommendations and standard guidelines of WHO report 2005. The cell cultures used for virus isolation include – SIRC and Vero. The cell cultures were incubated for a period of seven days and in twenty TC wells changes were seen at the end of five days as shown in figures 7a & b. All the 335 specimens were subjected to virus isolation using both the cell lines and the results obtained were compared. The summary of the results obtained using these cell lines have been tabulated in table 8. IIF staining was standardized by varying the dilutions of both primary and secondary antisera used. The primary was standardized as 1:100 and secondary as 1: 50 as shown in figure 8. Although the IIF staining technique was standardized, direct application of this technique onto the clinical specimens did not detect RV, indicating the low sensitivity of this technique. Twenty isolates from 335 specimens were obtained using SIRC cell line, whereas only 9 was obtained using vero cell line. These twenty isolates were confirmed by both IIF and nRT-PCR techniques.

Figure 07a

Photographs of standard strain of RV in Vero cell line



Un Infected Vero cell line

Monolayer of Vero cells with HPV77 standard strain of RV

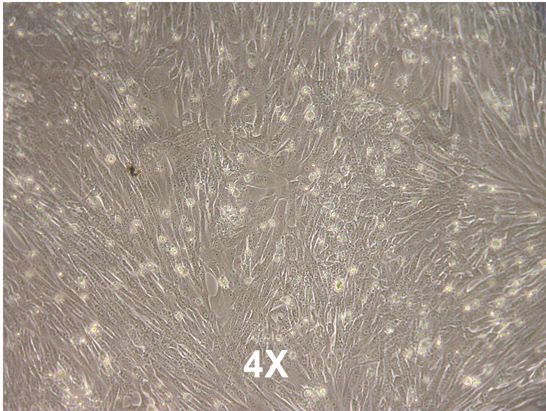
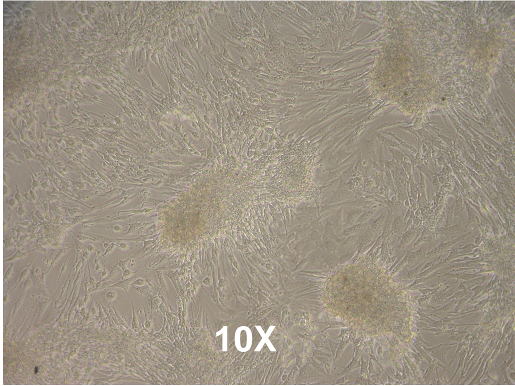
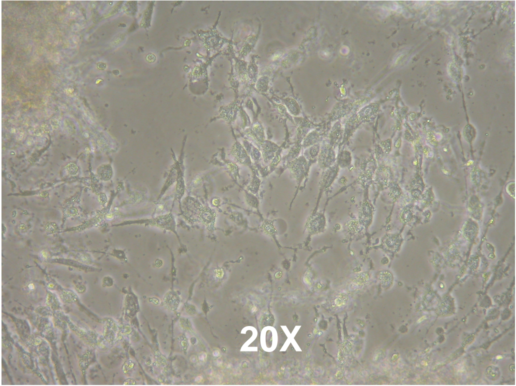


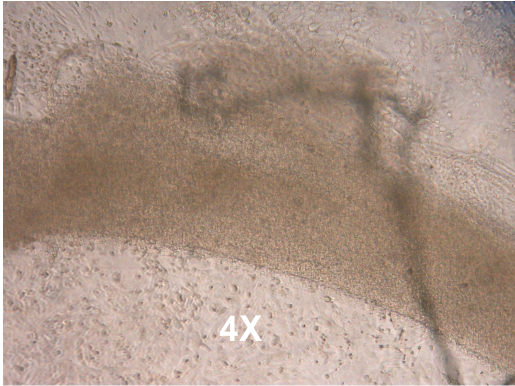
Figure 07b

Photographs of RV isolates in SIRC cell line

Monolayer of SIRC cells inoculated with Lens aspirate specimen







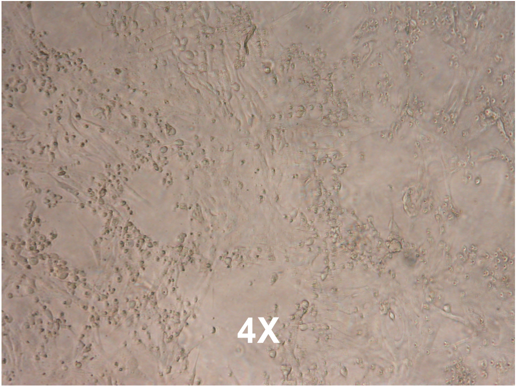


Figure 08

In direct Immunofluorescence staining for RV

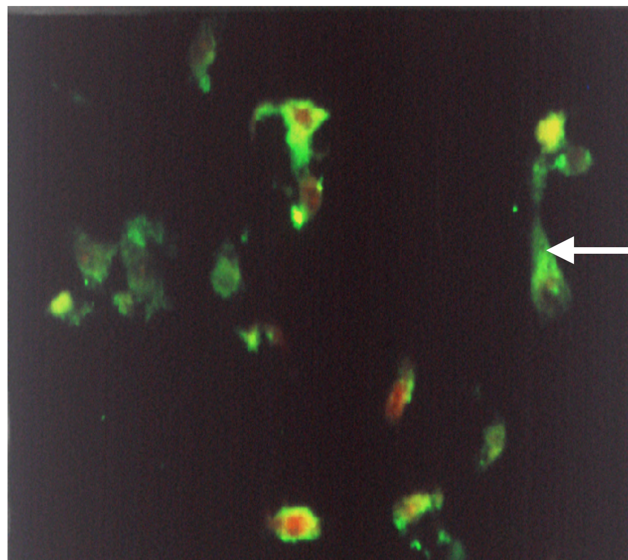


Table 8: Shows the results of RV isolation from 335 lens aspirates and six amniotic fluid specimens inoculated into SIRC and vero cell cultures

S. No	Group	Clinical specimen	SIRC	Vero
1	A	Lens aspirates	07	04
2	B	Lens aspirates	06	02
3	C	Lens aspirates	05	02
4	D	Amniotic fluid	02	01
		Total	**20**	**09**

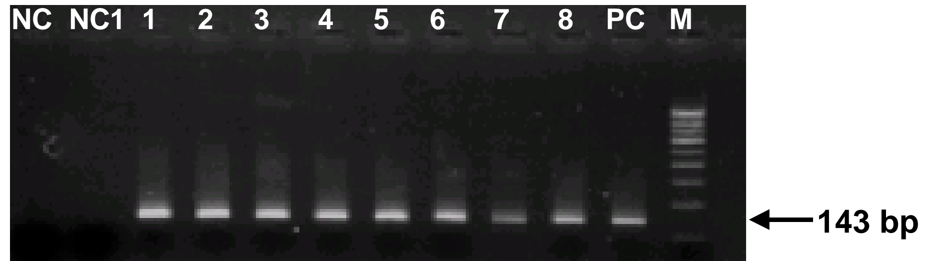
Note: RV strains isolated in vero were also isolated in SIRC indicating the increased sensitivity of SIRC for RV isolation.

5.4 Results of Genotyping:

The twenty isolates were amplified using the primers targeting the E1 gene of RV. The amplified products were verified by agarose gel electrophoresis as shown in figure 9. The amplicons were analyzed by DNA sequencing (figure 10). The genotyping was done using the MultAlin software version 5.4.1. The sequences were compared with the Cendehill strain and Therein strain belonging to Genotype I of RV genome. The BRD II strain represented the genotype II. The percentage homology was also calculated and tabulated in table 9. Eighteen strains belonged to genotype I and two strains belong to Genotype II of RV (figure 11). The two strains belonging to Genotype II were obtained from Chinese nationals.

Figure 09

Agarose gel photograph showing the amplified products of Rubella virus on the culture harvests



Lanes NC, NC1 – Negative controls

Lanes 1 – 8 – Rubella virus RNA from lens aspirate inoculated SIRC cell line

Lane P – HPV 77 RNA of Rubella virus

Lane M - (φ X 174 DNA/ *Hinf I* digest)

Figure 10

Results of DNA sequencing on the amplified products of nRTPCR positive lens aspirate specimens

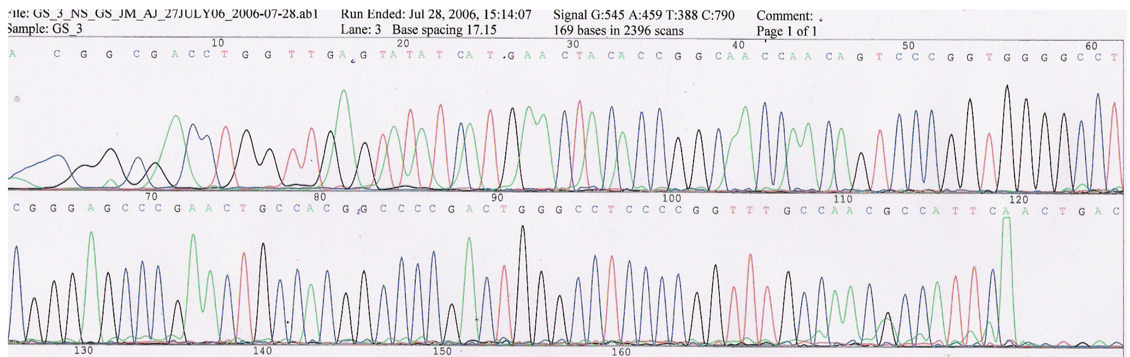


Table 09: Results of genotyping of RV isolates

Isolate ID	Percentage homology with Genotype I	Percentage homology with Genotype II	Genotype
001	75	25	I
002	75	25	I
003	63	37	I
004	63	37	I
005	63	37	I
006	75	25	I
007	75	25	I
008	25	75	II
009	75	25	I
010	63	37	I
011	75	25	I
012	75	25	I
013	75	25	I
014	75	25	I
015	25	75	I
016	87	13	II
017	63	37	I
018	63	37	I
019	75	25	I
020	75	25	I

Note: As per guidelines of WHO report 2005.

Figure 11a

Nucleotide sequence comparison of RV isolates with the genotypes using Multiple sequence alignment software

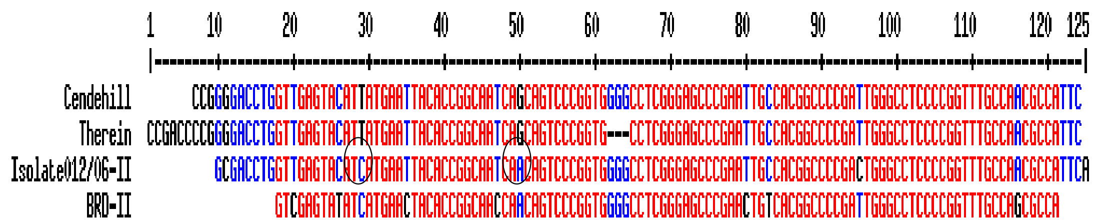
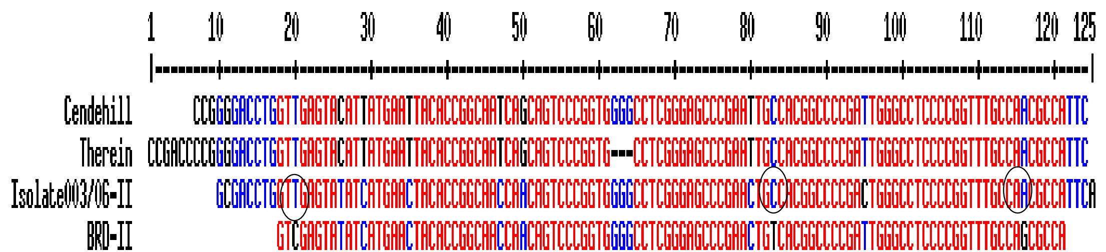


Figure 11b

Nucleotide sequence comparison of RV isolates with the genotypes using Multiple sequence alignment software

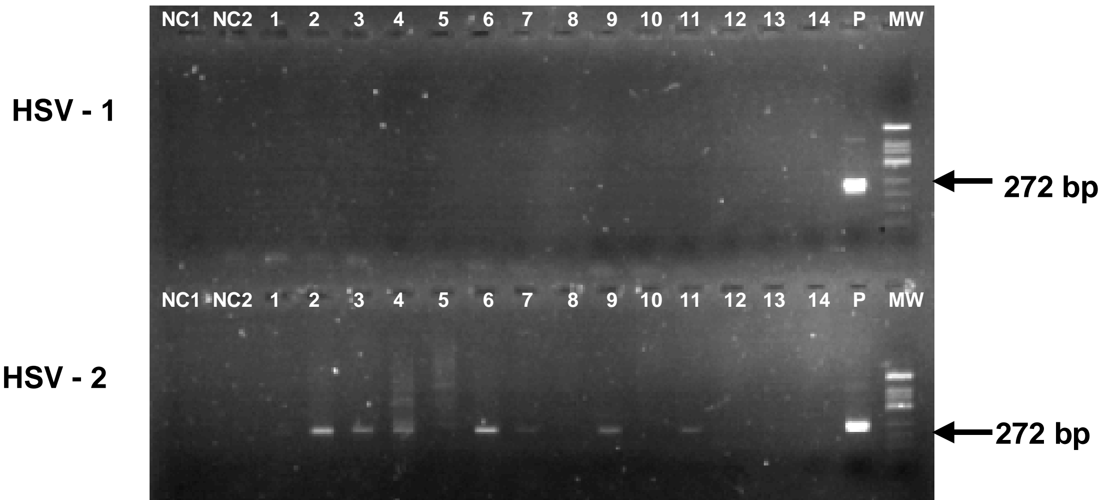


5.5 Results of nucleic acid based amplification techniques for the determination of viral etiology in congenital cataract:

Nucleic acid amplification based methods were applied on fifty lens aspirates for association of Herpes Simplex Virus (HSV), Cytomegalovirus (CMV), Varicella Zoster virus (VZV) and Rubella virus (RV). Standardized Polymerase chain reaction for the detection of HSV, CMV and VZV DNA was applied on lens aspirates obtained from congenital cataract in the age group of 6days to 9 months after birth. nRT-PCR was used for the detection of RV RNA. The PCRs applied include a snPCR for the detection of HSV, snPCR for the detection of VZV and nPCR for the detection of CMV. nRT-PCR was applied for the detection of RV. snPCR detected the presence of HSV in 9 (18%) lens aspirate specimens. The serotype detected was HSV 2 as shown in figure 12. nRT-PCR detected the presence of RV genome in 9 (18%) additional lens aspirates. None of the fifty lens aspirates tested showed the presence of both VZV and CMV. Hence a total of 18 (36%) of fifty lens aspirate specimens were positive for a viral etiology. No viral co - infections were noted in our study. We failed to isolate HSV from any of the lens aspirate positive specimens hence we confirmed the positivity by Real Time PCR. The viral particles were quantitated by comparison with the standards. Figure 13 shows the Real time data of the specimens in comparison with the standards. Table 10 compares the results obtained in snPCR and the Real time PCR.

Figure 12

Agarose gel electrophoretogram showing the application of snPCR onto lens aspirate specimens



Lanes NC1, NC2 – Negative controls
Lanes 1 – 14 – Lens aspirate specimens
Lane P – Respective HSV 1 & 2 Positive control
Lane M - φ X 174 *Hinf I* digest Molecular weight marker

Figure 13

Real time PCR on Lens aspirate samples

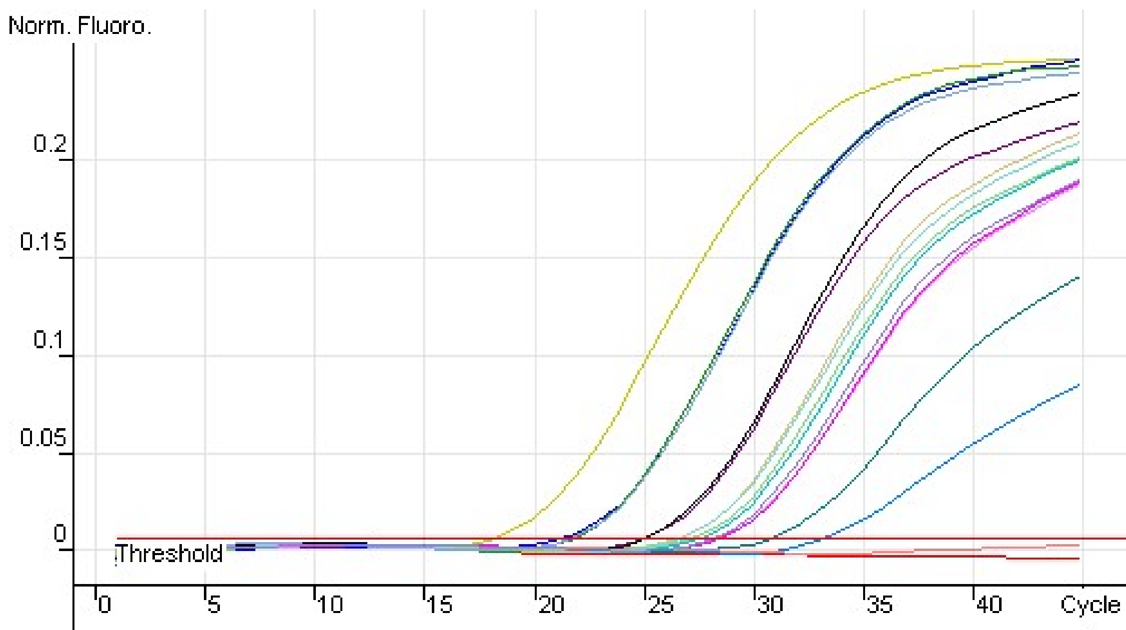


Table 10: Results of snPCR, and Real Time PCR for the detection and quantification of HSV in Lens aspirate specimens

S. No	snPCR	Real Time PCR	Copies / ml
1	Positive	Positive	30,836
2	Positive	Positive	87,19,938
3	Positive	Positive	13,36,90
4	Positive	Positive	11,47,758
5	Positive	Positive	23,9026
6	Positive	Positive	4,023,56
7	Positive	Positive	3,052,24
8	Positive	Positive	3,89,757
9	Positive	Negative	--------

5.6 Serological results for the determination of viral etiology in congenital cataract:

Serological investigations were done for these fifty patients using the serum sample. Serological diagnosis was done using commercially available ELISA kits BIO ELISA procured from BIOKIT, Barcelona, Spain, as per the instructions provided in the manual for the presence of IgG and IgM antibodies against RV, HSV and CMV. Considering the results of antibodies against CMV, IgM antibodies were not detected in any of the 50 patients, in 27 patients IgG antibodies were detected. Table 11 summarizes the results obtained in this study in comparison with the serological results.

Table 11: Shows the results of virus isolation & PCR on lens aspirates & serology for the association of RV, HSV & CMV with congenital cataract in 50 patients

I.D No.	Rubella virus				Herpes simplex virus			
	ELISA		nRT-PCR on lens aspirates (9 patients)	Virus isolation (6 patients)	ELISA			snPCR on lens aspirates (9 patients)
	IgG	IgM			IgG HSV 1	IgG HSV 2	IgM	
002 / 05	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**HSV 2**
005 / 05	**Pos**	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg	Neg
006 / 05	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg
007 / 05	**Pos**	Neg	Neg	Neg	Neg	Neg	Neg	**HSV 2**
008 / 05	**Pos**	Neg	Neg	Neg	**Pos**	Neg	Neg	**HSV 2**
010 / 05	Neg	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg
011 / 05	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**HSV 2**
012 / 05	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**HSV 2**
019 / 05	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**HSV 2**
023 / 05	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg
026 / 06	Neg	Neg	Neg	Neg	Neg	Neg	Neg	**HSV 2**
027 / 06	**Pos**	Neg	**Pos**	**Pos**	Neg	Neg	Neg	Neg
028 / 06	**Pos**	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg
032 / 06	**Pos**	Neg	Neg	Neg	**Pos**	Neg	Neg	**HSV 2**
039 / 06	**Pos**	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg	Neg
040 / 06	**Pos**	**Pos**	**Pos**	Neg	Neg	Neg	Neg	Neg
041 / 06	**Pos**	Neg	**Pos**	Neg	Neg	Neg	Neg	Neg
044 / 06	Neg	Neg	Neg	Neg	**Pos**	Neg	Neg	**HSV 2**

NOTE:

- **CMV DNA was not detected in any of the 50 lens aspirates, although serologically anti CMV IgG was detected in 27 patients.**
- **HSV 2 DNA was present in 9 lens aspirates; anti HSV 2 IgM & IgG antibodies were not detected in any of the patients.**

5.7 Results of standardization of snPCR for the rapid detection and identification of HSV serotypes in culture negative intraocular aspirates:

Twenty-one intraocular fluids collected from 19 patients (16 with acute retinal necrosis (ARN), 2 with viral retinitis, and one each of progressive outer retinal necrosis (PORN), CMV retinitis and optic neuritis) were included in the study. Seventeen aqueous humor (AH) and 4 vitreous aspirates (VA) were collected. Dual specimens of AH and VA were collected from 2 patients with ARN.

Uniplex PCR for DNA polymerase gene of HSV:

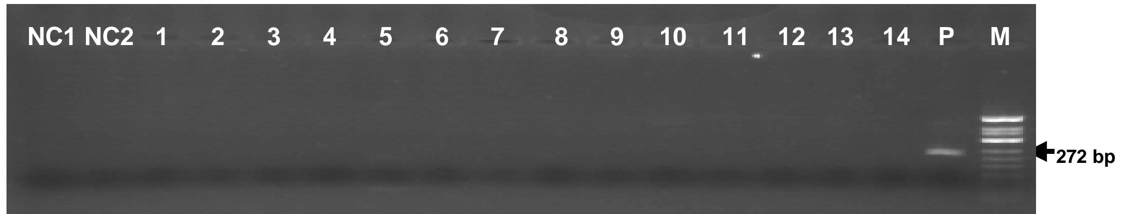
Of the 21 intraocular fluids tested, HSV DNA was detected in four. These were from AH and VA of two patients with clinical diagnosis of ARN. HSV DNA was not detected by uPCR from all other 17 intraocular clinical specimens.

Specificity and sensitivity of snPCR for HSV 1 and 2:

The snPCR for Glycoprotein D gene was standardized and was specific for HSV DNA. It did not amplify the DNA from other organisms, which included viral, bacterial, fungal and the parasite *Toxoplasma gondii* (figure 14). The results of the specificity of the primers with the clinical isolates exactly coincided with the serotyping done earlier proving their specificity (Madhavan et al., 2003). The sensitivity of the primers was determined as 0.02 attograms of DNA of both HSV 1 and 2 (figure 15).

Figure 14

Agarose gel electrophoretogram showing the specificity of the gD gene primers used in the snPCR for the detection of HSV genome



Lanes NC1, NC2 – Negative controls

Lanes 1 – 12 – DNA from other infective organisms

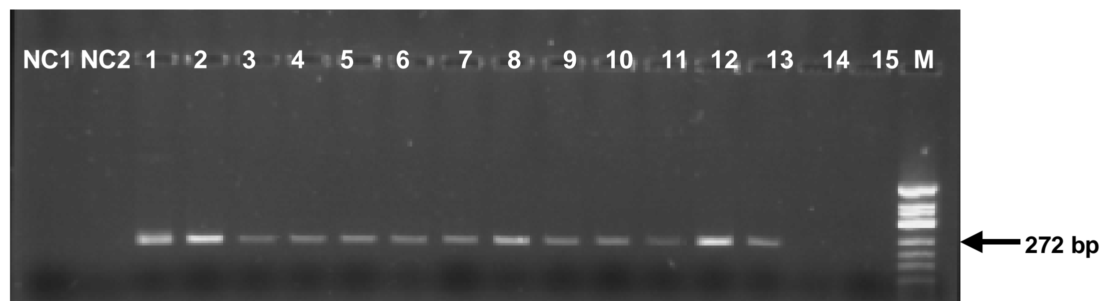
Lane 13, 14 – Human DNA

Lane P – Positive control HSV 1 DNA

Lane M - φ X 174 *Hinf I* digest Molecular weight marker

Figure 15

Agarose gel electrophoretogram showing the sensitivity of the gD gene primers used in the snPCR for the detection of HSV genome



Lane NC 1, NC2 – Negative controls

Lane 1 – 15 – Log $_{10}$ dilutions of HSV DNA

Lane M - φ X 174 *Hinf I* digest Molecular weight marker

Table 12: **The results of snPCR applied onto 21 intraocular fluids collected from patients with Acute retinal necrosis**

S. No	Clinical specimen	Results of uPCR	Results of snPCR
1∗	AH	Positive	Positive / HSV 1
2∗	VA	Positive	Positive / HSV 1
3•	AH	Positive	Positive / HSV 2
4•	VA	Positive	Positive / HSV 2
5	AH	Negative	Positive / HSV 2
6	AH	Negative	Positive / HSV 1
7	AH	Negative	Positive / HSV 2

AH: Aqueous humor **VA**: Vitreous aspirate

∗ - Dual specimen collected from Patient 1
• - Dual specimen collected from Patient 2

Application of the standardized snPCR onto intraocular aspirates:

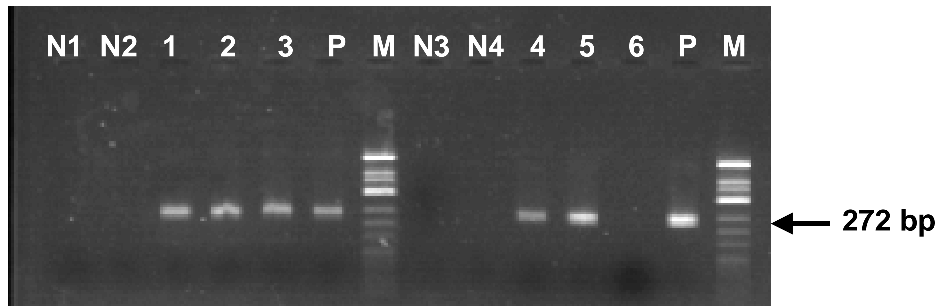
This standardized PCR was applied onto the 21 intraocular fluids and HSV DNA was detected in 7 specimens (31.8%). The results of snPCR in comparison with uPCR targeting the DNA Polymerase gene are shown in table 12. The seven positives include the four of uPCR. snPCR differentiated 3 of the 7 as HSV 1 and other 4 as HSV 2. All the seven positives were from patients with clinical diagnosis of ARN. The results of the snPCR are shown in Figure 16.

DNA sequencing of the snPCR amplified products:

The seven positives of the snPCR were DNA sequenced (ABI prism 300) and the results are shown in Figures 17a &b. The sequence that was obtained was submitted to BLAST search tool of NCBI to analyze the percentage homology with the standard strains of HSV 1 and 2 to serotype them. The results clearly showed that three positive i.e dual specimens from a single patient of ARN and AH from another patient of ARN were identified as HSV 1 serotype. The other four positives were identified as HSV 2, which included a dual specimen of AH and VA collected from a single patient of ARN and AH collected from two patients with the same clinical diagnosis. All the seven snPCR products showed 100% homology with that of the standard strains of HSV 1 and 2 on DNA sequencing.

Figure 16

Agarose gel electrophoretogram showing the amplified products of Herpes simplex virus 1 and 2 on culture negative intraocular fluids



Lanes N1, N2, N3 and N4 - Negative controls

Lane 1, 3 – Aqueous humor (AH) positive for HSV 1

Lane 2 – Vitreous humor (VA) positive for HSV 1

Lane P – Positive control HSV 1 & HSV 2

Lane 4 – AH specimen positive for HSV 2

Lane 5 – VA specimen positive for HSV 2

Lane 6 – AH specimen Negative for HSV 2

Lane M - (φ X 174 DNA/ *Hinf I* digest)

Figure 17a

Results of DNA sequencing on the amplified products of snPCR positive for Herpes simplex virus 1 in culture negative intraocular specimens

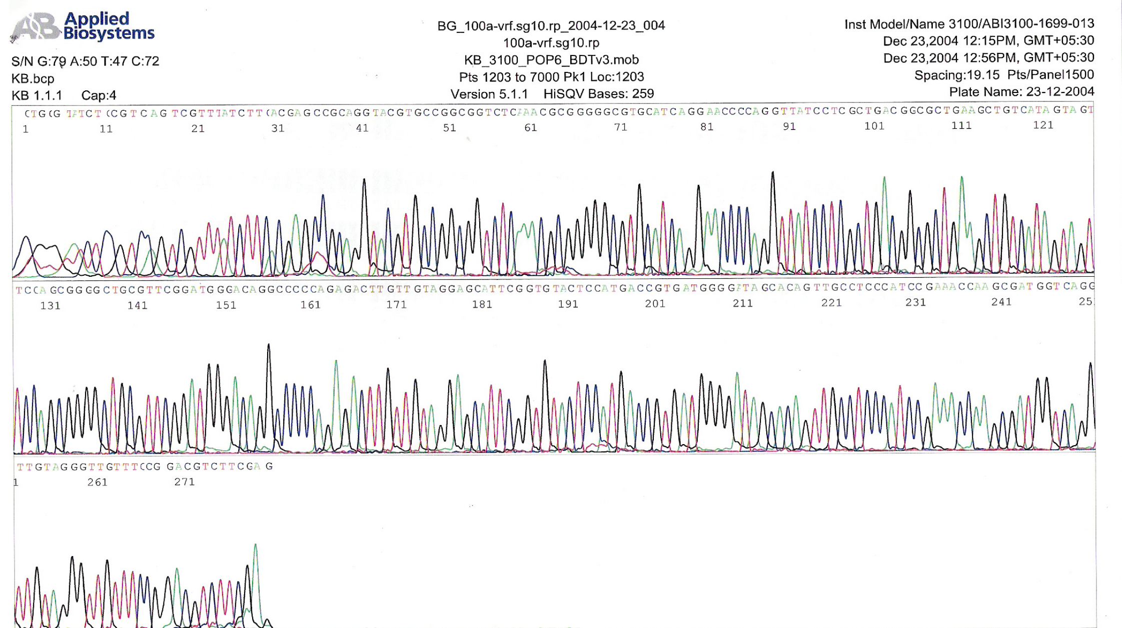
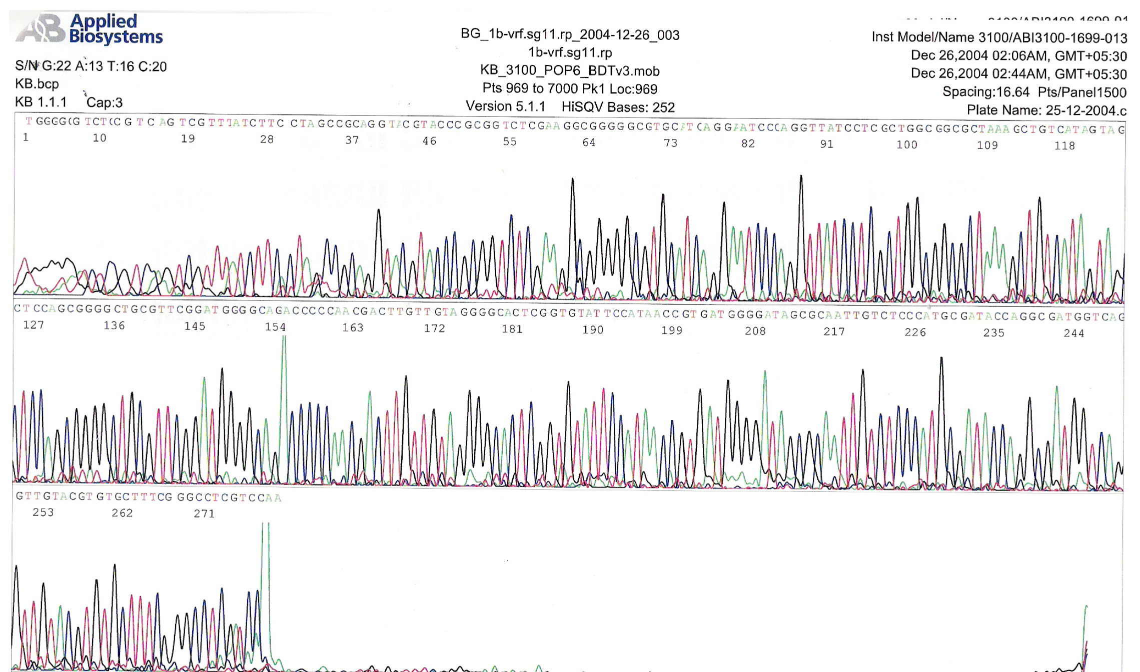


Figure 17b

Results of DNA sequencing on the amplified products of snPCR positive for Herpes simplex virus 2 in culture negative intraocular specimens



nested PCR for CMV:

CMV DNA was detected in two (9.5%) of the intraocular fluids (2 AH) collected from 2 patients with CMV retinitis and ARN respectively. The PCR was sensitive enough to detect 0.002 femtograms of CMV DNA as quantitated by us earlier (Priya et al., 2002; Mitchell et al., 1995). Presence of a band in the 128bp region indicated positivity.

seminested (sn) PCR for VZV:

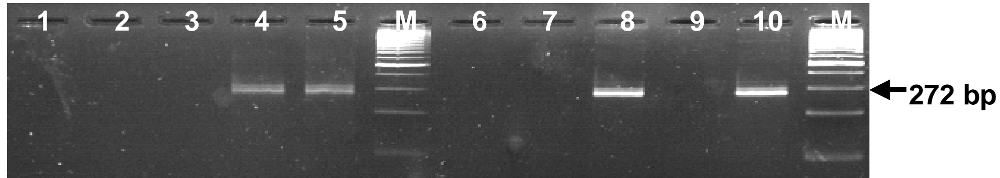
The snPCR targeting the immediate early 63 gene of VZV detected five positives (23.8%) from 21 intraocular fluids. Of the five positives, four patients had ARN and one was with PORN in which VZV DNA was detected. The snPCR was sensitive enough to detect 20 femtograms of VZV DNA as described and quantitated by us earlier (Priya et al., 2003).

5.8 Application of snPCR for the detection of HSV in different types of clinical specimens:

A total of 546 clinical specimens (359 Cerebrospinal fluid specimens, 152 ocular specimens, 11 swabs collected from oral lesions, 9 peripheral blood specimens, 7 genital swabs, 3 swabs collected from skin blisters and five miscellaneous specimens consisting four BAL specimens and a tracheal aspirate) collected from 540 patients were tested for the presence of HSV genome. 21 (3.84%) specimens were positive for HSV 1 genome, of which 6 were ocular specimens, 10 were CSF specimens, 4 oral specimens, and a scarping from skin blister; 31 (5.67%) specimens were positive for HSV 2 serotype. A majority of 21 specimens of CSF were positive for HSV2 serotype followed by 5 ocular specimens, 2 blood specimens and one specimen each of oral swab, genital swab and scraping from skin blister. The agarose gel photograph showing the application of snPCR on to clinical specimens is shown in figure 18. The serotype specificity of these positive specimens were further verified by the application of PCR based RFLP. The results of PCR – RFLP were in concordance with that of the results of snPCR (figure 19).

Figure 18

Agarose gel electrophoretogram showing the amplified products of HSV- 1 & 2 from clinical specimens

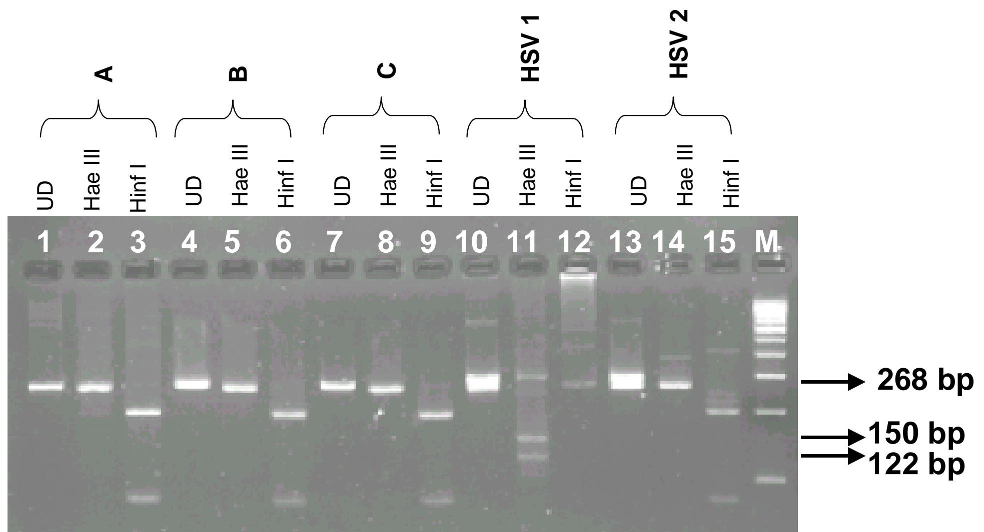


Lanes			Lanes		
1.	Negative control 1	- Negative	6.	Negative control 1	- Negative
2.	Negative control 2	- Negative	7.	Negative control 2	- Negative
3.	Aqueous tap	- Negative	8.	Aqueous tap	- Positive
4.	Vitreous aspirate	- Positive	9.	Vitreous aspirate	- Negative
5.	Positive control	- Positive	10.	Positive control	- Positive

M - 100 bp DNA ladder

Figure 19

Restriction enzyme digestion of PCR amplified products of HSV with *Hinf I* and *Hae III*



A, B and C - PCR amplified products from lens aspirate specimens
UD – Undigested PCR amplified product

5.9 Results of IIF staining for the detection of HSV from different clinical specimens:

IIF staining was performed from corneal scrapings, swabs collected from genital ulcers, swabs collected from oral lesions, and cerebrospinal fluid specimens. IIF staining revealed the presence of HSV in 1 corneal scraping, wherein the snPCR was also positive. Of the seven genital swabs tested, IIF detected HSV antigen in a single specimen alone. None of the CSF samples revealed the presence of HSV antigen and this could be due to the presence of minimal cellular material. None of the oral swab specimens revealed the presence of HSV antigen.

5.10 Results of application of nPCR for the detection of Ad from CS specimens:

157 conjunctival swab specimens collected from 115 patients suffering from epidemic acute hemorrhagic conjunctivitis were included in the study. The patients were asked to come for three follow up study. The initial visit was considered as day1 and the 3rd day visit as day 2 and 6th day visit as day 3. Only 18 patients came for the day 2 visit and 12 patients for the day 3 visit. nPCR detected the presence of Ad in 19 conjunctival swab specimens collected from 18 patients, of which a single patient had come for the second follow up and was continuing to be positive for Ad. The primers are designed to target the highly conserved region of the hexon gene so that it can amplify all the serotypes of Ad.

Results of DNA sequencing on Ad positive PCR amplified products:

Since the nPCR cannot distinguish between the serotypes of Ad, further characterization was done using the DNA sequencing technique. All the 19 positives were sequenced and the sequence was submitted to BLAST search tool of NCBI to calculate the percentage homology with serotypes of Ad. 98% homology was seen with serotype 8 of Ad.

Results of virus isolation using cell cultures:

Using HEp - 2 cell culture, Ad was isolated from 12 specimens collected from 18 patients. The characteristic CPE of grape like clusters were seen and the infected cells were harvested and IIF staining was performed for

further confirmation. IIF staining revealed the presence of Ad in all the 12 specimens.

5.11 Standardization of nRT-PCR for the detection of human enterovirus in conjunctival swab specimens collected from EAHC patients:

The nRT-PCR was standardized using the standard strain of CA24.

Specificity: The optimized nRT-PCR for 5' non - coding region was specific for enteroviruses since there was no amplification of RNAs from infective agents namely HSV, *Chlamydia trachomatis*, Adenovirus and human RNA included in the reactions.

Sensitivity: The sensitivity of the primers was determined using the RNA extracted from the each of the 10 log dilutions. The standardized nRT-PCR was set up for each of the dilutions and the detection limit was found to be 12 femtograms (fg). The agarose gel photograph showing the sensitivity of the primers is depicted in figure 20.

Results of application of standardized nRT-PCR onto CS specimens:

A total of 157 clinical specimens collected from 115 patients with EAHC, were rapidly screened for the presence of human EVs. The 115 patients were categorized into three groups namely –

Group I consists of 89 patients who had come only for a single visit.

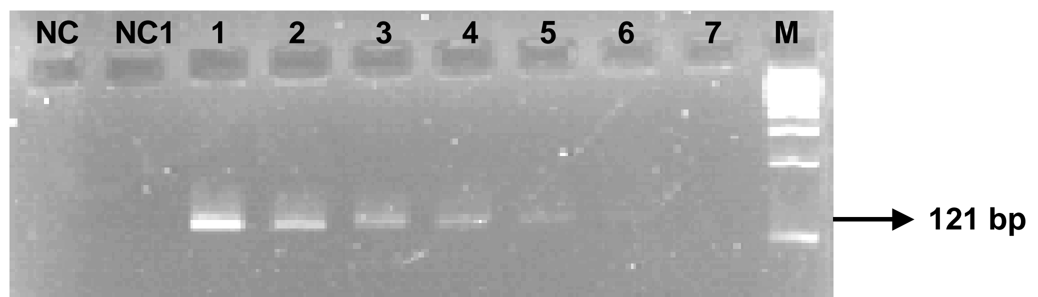
Group II consists of 18 patients who came for two visits.

Group III consists of 12 patients who came for all the three visits.

In the group I 47 CS specimens collected from 47 patients with EAHC were positive for EV. Twelve of the 18 patients in group II were positive for EV. All these patients were positive for EV even in their second visit.

Figure 20

Agarose gel electrophoretogram showing the sensitivity of the primers used in the nRT-PCR for the detection of Human Enteroviruses



Lanes NC - Negative control IInd Round

Lane NC1 – Negative control Ist Round

Lane 1 – Neat Polio virus type 1 RNA

Lanes 2 – 6 Log $_{10}$ dilutions of Polio virus type 1 RNA

Lane M - φ X 174 *Hinf I* digest Molecular weight marker

All the twelve patients in group III were found to be positive for EV. Eleven patients were positive only till the day 2 visit and all of them became negative in their day 3 visit. Only a single patient was positive in all the three visits for EV. The application of nRT-PCR for the detection of EV on CS specimens is shown in figure 21.

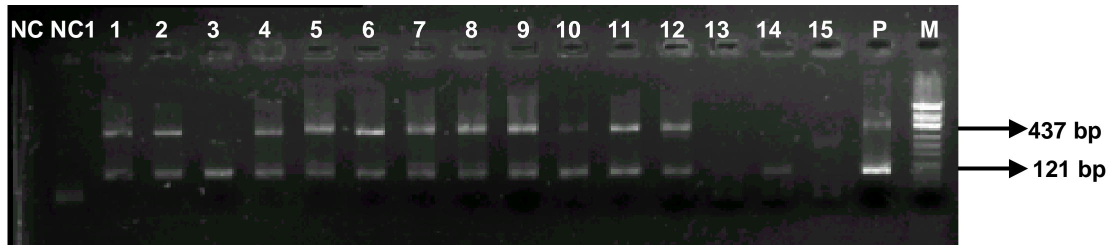
There are no viral co infections reported in our study.

Results of virus isolation using cell cultures:

Three cell cultures were used for EV isolation. HEp 2, HeLa, Vero cell cultures were compared for their sensitivity of isolation of EVs. Fifty EV isolates were obtained using HeLa cell culture; thirty-two isolates using HEp – 2 and eighteen using vero cell culture. The isolates obtained from HeLa cell culture were also obtained from vero and HEp –2 and no new isolates were obtained in the other two cell cultures indicating that HeLa cell culture is sensitive for EV isolation. These isolates were further characterized by DNA sequencing and found to be CA24v.

Figure 21

Agarose gel electrophoretogram showing the application of nRT- PCR onto conjunctival swab specimens for the detection of Human Enteroviruses



Lanes NC, NC1 – Negative controls

Lanes 1 – 15 – Conjunctival swab specimens

Lane P – RNA from Polio virus type 1

Lane M - φ X 174 *Hinf I* digest Molecular weight marker

5.12 Standardization of uRT-PCR for the detection of CA24 variant in conjunctival swab specimens collected from EAHC patients:

The uRT-PCR was standardized using the standard strain of CA24.

Specificity: The optimized uRT-PCR for VP1 region was specific for CA24 variant (CA24v) since there was no amplification of RNAs from infective agents other than CA24v and human RNA included in the reactions as shown in figure 22.

Sensitivity: The sensitivity of the primers was determined using the RNA extracted from the each of the 10 log dilutions. The standardized uRT-PCR was set up for each of the dilutions and the detection limit was found to be 12 femtograms (fg). The agarose gel photograph showing the sensitivity of the primers is depicted in figure 23.

Results of application of standardized uRT-PCR onto CS specimens:

A total of 157 clinical specimens collected from 115 patients with EAHC, were analyzed for the presence of CA24v. The 115 patients were categorized into three groups namely –

Group I consists of 89 patients who had come only for a single visit.

Group II consists of 18 patients who came for two visits.

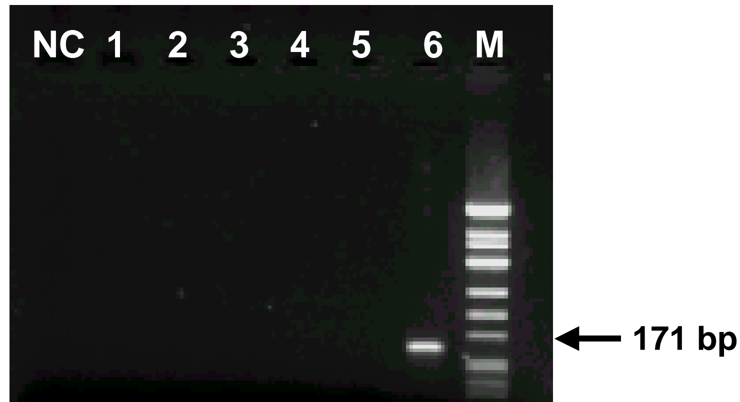
Group III consists of 12 patients who came for all the three visits.

In the group I 29 CS specimens collected from 29 patients with EAHC were positive for CA24v. Six of the 18 patients in group II were positive for CA24v. All these patients were positive for CA24v even in their second visit. Of the 12 patients in group III, eight patients were positive.

Seven patients were positive only till the day 2 visit and all of them became negative in their day 3 visit. Only a single patient was positive in all the three visits for CA24v. The application of uRT-PCR on CS specimens is shown in figure 24. There are no viral co infections reported in our study.

Figure 22

Agarose gel electrophoretogram showing the specificity of the VP1 primers used in the uRT-PCR for the detection of CA 24 variant



Lanes NC – Negative control

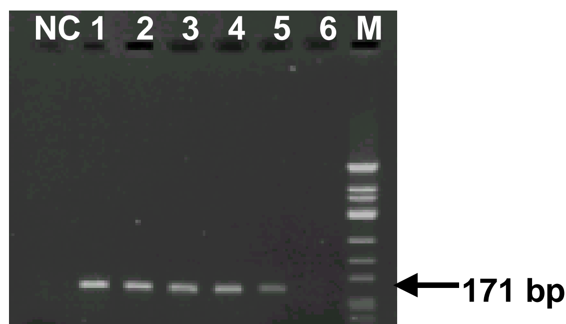
Lanes 1 –5 – DNA from other infective organisms causing conjunctivitis

Lane 8 – RNA from CA24v standard strain

Lane M - φ X 174 *Hinf I* digest Molecular weight marker

Figure 23

Agarose gel electrophoretogram showing the sensitivity of the VP1 primers used in the uRT-PCR for the detection of CA 24 variant



Lanes NC - Negative control

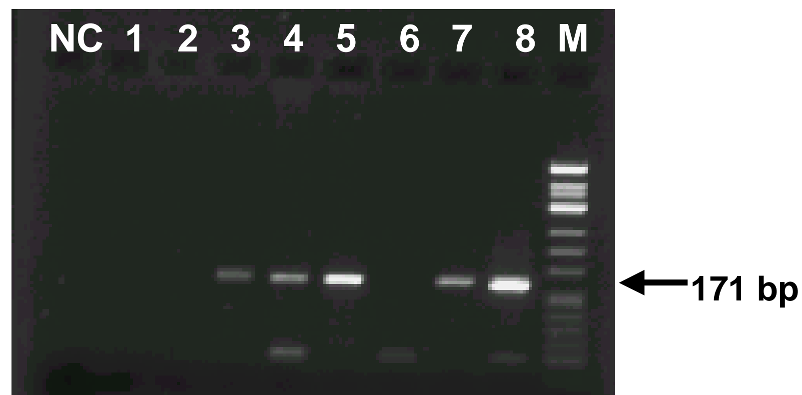
Lane 1 – Neat CA24v RNA

Lanes 2 – 6 Log $_{10}$ dilutions of CA24v RNA

Lane M - φ X 174 *Hinf I* digest Molecular weight marker

Figure 24

Agarose gel electrophoretogram showing the application of uRT- PCR onto conjunctival swab specimens for the detection of CA 24variant



Lanes NC – Negative control

Lanes 1 – 7 – DNA from Conjunctival swab specimens

Lane 8 – RNA from CA24v standard strain

Lane M - φ X 174 *Hinf I* digest Molecular weight marker

5.13 Results of DNA sequencing on CA24v PCR amplified products:

The sequenced samples were analyzed and the sequence was submitted to the BLAST search tool of NCBI. 97% homology was obtained with CA24v. The variations between the positive samples and the standard strain were analyzed using the MultiAlin software. 8.6% variation was seen between the standard strain and the epidemic strain of CA24v (Figure 25).

Figure 25

Nucleotide sequence comparison of CA 24 positive samples with standard strain using Multiple sequence alignment software

```
           1       10        20        30        40        50        60        70        80        90        100       110       120       130
           |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
  unknown  GCTGCAGTTAGTGCGG-GCCC-----CCCCGCGAGTCG-ATTTAAAAGCAAGCGGCGCGGACCGAGACGCGCGCCGCTAAACGCGGAAAGGCCGTGAAAG--CTGTAATGCATTGGTAATAACGG---TG
 standard  GCACAAGGCATTGAGGAGACCATTGACACCGTCATTAGCAATGCACTACAACTGTCACAACCTAAACCGCA-GAAACAACTCACGGCCCAATCGACACCGTCCACCAGTGGAGTGAATTCACAGGAAGTG
Consensus  GCacaAGgcAgTGaGG.GaCC.....CaCCGccAgTaG.AaTgaAaaaCAAccGgCaCaaaCcaAaaCGCa.GaaaCaAaaCaCGGaaaaacCGacAaaG..CaccAaTGcAgTGaaaacAaaGG...TG

           131     140       150       160       170 174
           |--------+---------+---------+---------+---|
  unknown  TCAATGGTCTCCTCAATG---CCTTGTGCACTTCCCAAAGAATA
 standard  CCTGCACTCACTGCGGTGGAGACTGGTGTATCCGGCCAGGCA
Consensus  cCaacacTCaCcgCaaTG...aCTgGTGcAcccccCaAaGaA..
```

6.DISCUSSION

The results of the methods of RNA extraction by various methods confirm that the standard GTC method, was more sensitive when compared to other two methods. The OD readings obtained were two to three times higher and purification was higher when compared to the other two methods. In case of cell free fluids, the QIAmp Viral RNA extraction kit was efficient in RNA extraction and GTC was not very efficient. In two urine samples, the RNA was not pure as urine contained lot of cellular debris and inhibitors. The results of the present nRT-PCR-based study on clinical specimens indicated association of RV with 12 % of 184 congenital cataract in patients of less than one-year age. In our earlier study (Malathi et al., 2001) over a period of 9 years on 70 patients with congenital cataract, the association of RV was 10 % based on RV isolation, which were identified by immunofluorescence method. In order to improvise the sensitivity of the detection rate, nRT-PCR has been optimized and applied for direct detection of the RV genome in lens aspirates, urine and peripheral blood leucocytes. The detection rate by nRT-PCR directly on clinical specimens was much higher when compared to virus isolation, as RV was isolated by conventional cell culture methods in only twenty clinical specimens compared to forty-two positive by nRT-PCR. SIRC was found to be much more sensitive for RV isolation than Vero. Trent J Bosma et al., 1995 applied RT-PCR on formalin fixed

tissues with detection rate of 53.3% whereas in our present study it was only 12 % though fresh lens aspirates were used. The nRT-PCR detected RV genome in 29 lens aspirates including the eighteen culture positive specimens and no false positive results were obtained. The isolates of RV were confirmed by direct DNA sequencing of the first round amplified product. Our results are comparable with that of those of the recent study at Madurai, south India, by Eckstein et al., 1996 where ELISA on the salivary secretions was used with 20% of cataract cases associated with congenital rubella. Angara et al., in 1987 have reported 21 % of RV associated congenital cataract in North India. These results may not strictly be comparable because of the differences in the laboratory techniques. In two of our patients, RV was isolated from the lens aspirate and RV RNA was detected from the all the three specimens viz. lens aspirate, peripheral blood specimen and urine specimen with both anti-RV IgG and IgM circulating in the blood indicating that RV antibodies did not prevent the virus multiplication in the patient. Although serological tests are supportive tests, direct detection of the viral RNA in clinical specimens by RT-PCR or by isolation would be the ideal choice for association of RV to congenital cataract. The whole procedure of RNA extraction along with the nRT-PCR needed just seven hours as against 7 – 14 days in case of virus isolation and moreover nRT-PCR was more sensitive compared to conventional virus isolation method. Virus isolation was less suitable since it needed expensive maintenance of cell cultures,

technically more demanding with results being available only after 3- 4 weeks. Failure to isolate the virus as happened in eleven of our nRT-PCR positive specimens could be attributed to low viral load in these specimens as suggested by Bosma et al., 1995. This was confirmed by the detection of the RV genome amplified product only in the second round. Use of nRT-PCR on lens aspirates appeared to be of more value than on the peripheral blood leucocytes and serology showed minimal role for such investigations. It is concluded that the nRT-PCR was a sensitive and specific technique for the detection of RV in the clinical specimens for congenital cataract patients.

As, the clinical diagnosis of RV is unreliable, rapid laboratory diagnosis of Rubella in pregnancy is critical to the decision for the Termination of Pregnancy (TOP) (Best et al., 1995). The risk of congenital infection may usually be estimated by establishing the gestational age at the time of maternal infection. However, it may be useful to attempt prenatal diagnosis of intrauterine infection under circumstances in which risk is difficult to establish, such as when there is no history of rash in the mother, maternal serology is inconclusive, infection has occurred between 13 and 20 weeks of gestation, or maternal reinfection is confirmed or suspected in the first trimester (Capner et al., 1991; Best et al., 1989). It is possible to make an intrauterine diagnosis by detection of RV in such specimens as chorionic villus sample (CVS) and Amniotic fluid (AF). DNA amplification techniques, such as PCR, may be regarded as ideal

techniques for detection of viral nucleic acids in these specimens as they are very sensitive and rapid (Donner et al., 1993; Hohfeld et al., 1994; Hohfeld et al., 1991; Terry et al., 1990). Detection of specific DNA in AF, which may be obtained at amniocentesis at about 16 weeks of gestation, has been found to be valuable for intrauterine diagnosis of congenital cytomegalovirus and toxoplasmosis (Donner et al., 1993; Hohfled et al., 1994; Hohfled et al., 1991; Ranko et al., 1991). When specimens obtained for prenatal diagnosis were tested, results of RT-PCR and virus isolation were in agreement tested by both techniques. Of the six specimens of amniotic fluids tested, RV RNA was detected in two samples and RV was also isolated. There is no "gold standard" for prenatal diagnosis of fetal rubella virus infection, and studies by many authors have also proved that RT-PCR is sensitive tool to detect the presence of RV infection (Bosma et al., 1995; Mace et al., 2004).

A number of genetically distinct groups of RV currently circulate in the world. The molecular epidemiology of RV will primarily be useful in the Rubella control activities for tracking transmission pathways, for documenting changes in the viruses present in particular regions over time, and for documenting interruption of transmission of RV (WHO 2005). Studies by Zheng et al., 2003a have shown by phylogenetic analysis that at least two genotypes of RV exist. Rubella genotype I (RGI) isolates predominate in Europe, Japan and western Hemisphere. RGII viruses are limited to Asia and Europe. RGI viruses were also present in

most of the countries where RGII viruses were isolated. Many authors have proved the methodology of PCR amplification and direct sequencing from the amplified product to extend the sequence information available on RV isolates (Katow et al., 1997; Zheng et al., 2003a; Zheng et al., 2003b; Frey et al., 1993). The E1 gene sequence has been used for genotyping and phylogenetic analysis of RV isolates by many authors (Zheng et al., 2003a; Frey et al., 1993; Frey et al., 1998; Zheng et al., 2003b; Reef et al., 2002). Use of entire E1 gene for genotyping results in excessive time as reported by Zheng et al., 2003a. In the present study, a small region of E1 gene within the molecular epidemiology window was used for the nucleotide analysis. The multiple sequence alignment tool was used for determining the genotypes of RV. Eighteen of the twenty isolates have been genotyped as belonging to RGI and two to RGII. The two strains belonging to Genotype II were obtained from Chinese nationals and the other eighteen strains were obtained from Indian Nationals. The earlier unpublished observations by the authors (Zheng et al 2003a and Frey et al., 1998) have shown that genotype II is concentrated in Asia, has been disproved in our study, since all these isolates were of Asian origin and 90% of the isolates belong to genotype I and only 10% belong to genotype II and these two strains belonging to genotype II have been obtained from patients of China. Although almost all of the strains and isolates were amplified in cell culture prior to sequencing, the nucleotide sequence of the RV genome has been shown to

be conserved during multiple passages *in vivo* and *in vitro* (Frey et al., 1993; Katow et al., 1997). There was a marked degree of sequence conservation among the isolates sequenced. Variability in nucleotide sequences between virus strains is due to combination of the existence of independent genetic lineages and the evolution overtime of individual lineages; the contribution of each varying among different viruses (Rota et al., 1992). At the nucleotide positions at which variation occurred, 19.1% showed transitions and12.5% showed transversions. The interstrain variation ranged from 0.1% - 0.9%. In this study the interstrain variation of the nucleotide sequence of the RV genome was, in general, as comparable as those of other RNA viruses (e.g., 0.5% – 7.2% in measles virus (Taylor et al., 1991; Rota et al., 1994); 1.2% - 5.8% in human parainfluenza virus type 3 (Coelingh et al., 1988), 0.1% - 12% in mumps virus (Yamada et al., 1989). Although some RNA viruses show a spectacular degree of sequence divergence, the limited degree of sequence divergence in the E1 coding region of RV strains is not atypical. Other viruses that exhibit interstrain variability in structural protein coding regions are measles upto 7.2% (Taylor et al., 1991); influenzae C virus 0.1 – 6.6% (Buonagurio et al., 1985). Hence we have proved that the Indian patients have RV genotype I rather than genotype II as reported by other authors (Zheng et al., 2003; Frey et al., 1988).

Based on virus isolation and serology, we had earlier reported the association of RV in 10 % of congenital cataract in our hospital-based

study (Malathi et al., 2001). In order to improvise the sensitivity of the virus detection, we optimized a nRT-PCR for the detection of the RV RNA and applied it onto lens aspirates from 50 congenital cataract patients. The study also included virus isolation method and snPCR for HSV and VZV and nPCR for CMV. Direct evidences of association of RV and HSV 2 were demonstrated in 18 (36 %) lens aspirates with RV in 9 (18 %) and HSV 2 in 9 (18 %) others. VZV was not detected in any of the lens aspirates tested. Among the 9 RV associated lens aspirates, the virus was isolated from 6 lens aspirates in tissue cultures. For the first time in India, such direct evidences of these virus associations with congenital cataract have been demonstrated. Most studies in India and elsewhere only serology was used for such associations and being an indirect method the results of serological tests should be carefully interpreted (Fomda et al., 2004; Gandhoke et al., 2005; Palihawadana et al., 2003; Karakoc et al., 2003). Analyzing the results of the serology of the present study, we have clearly shown that anti RV IgM antibodies were present only in 3 of the 9 RV positive patients thus indicating that presence of anti RV IgM antibodies need not be considered as the hallmark of congenital rubella syndrome. The possible explanation for this could be that the primary infection of the lens would have been in central lens fibres with RV at a stage when immunological apparatus had not attained maturity to react to the viral antigen. Earlier studies have shown that the lenses from the first trimester rubella infected foetuses showed pyknotic nuclei, cytoplasmic

vacuoles, and inclusion bodies in the primary lens cells and retardation of lens development. Late changes included degeneration of some primary lens fibres and evidence of active disease in the newly developing equatorial lens fibre cells, indicating chronic infection (Webster et al., 1998). Direct detection of the viral RNA in clinical samples can identify almost all cases of intrauterine RV transmission within 24-48 hours after sampling (Mace et al., 2004). nRT-PCR was shown as more sensitive and rapid compared to conventional method of virus isolation and serology in this study. Regarding HSV infection, 9 lens aspirates showed the presence of HSV 2 DNA, although serologically antibodies against HSV 2 were not present in any of these patients. Since HSV 2 commonly causes genital disease, it is possible that a perinatal infection could have occurred when the baby passed through the infected birth canal or could be a congenital infection, as a result of ascending infection from the infected cervix of the mother. As explained by Cibis *et al* possible explanation could be that the virus entered the lens at the time of first trimester and persisted throughout the fetal development period, and hence the fetal immune system considered it as self-antigen. As shown in various studies, newborns typically acquire HSV 2 during passage through an infected birth canal, but transplacental spread accounts for a small proportion of cases. About 70 – 85 % of HSV infected infants are born to woman who have no history of genital herpes and are asymptomatic at the time of delivery (Brown et al., 2005; Whitley et al., 2004). Although our results correlate

well with studies in early 1970 and 80s, the recent study by Raghu *et al* reported the association of HSV 1 DNA (Raghu et al., 2004). Some studies suggest that cataracts not only progress but may also develop after birth (Boger et al., 1980; Townsend et al., 1975). Although we could detect the presence of the HSV 2 viral DNA, we failed to isolate HSV from all the 9 positive patients, indicating there was no active viral replication taking place in the infected infant although the real time data indicated the presence of as viral particles ranging from 30,836 – 87,19,938 viral copies / ml. The virus had probably left an imprint of its DNA during its infection in the lens causing congenital cataract before it had become inactive. Our study indicated that serology had a minimal or no role in identifying the causative virus in congenital cataract. Majority of the patients showed antibodies in IgG class against the three viruses studied and these probably had passed through placenta from the mother. Although CMV is said to be the cause of most congenital infections in the world, the present study clearly indicated that this virus has no role to play in the development of cataract in the 50 patients investigated. Serology indicated presence of anti CMV IgG in 27 patients and these might be due to passive transfer from the mother through the placenta. We conclude that that nucleic acid amplification tests in the form of PCR may be of value in confirming the aetiology of congenitally acquired viral infection. Serology appears to play a minimal or no role in identifying the pathogen. Detection of HSV 2 DNA in as many number of lens aspirates as that of RV

indicated its important role in the causation of congenital cataract in patients attending our hospital. CMV and VZV appeared to have no relationship with congenital cataract in these 50 patients.

The association of ARN, PORN and viral retinitis with herpes viral etiology has been well documented by many authors (Priya et al., 2003; Fox et al., 1991; Knox et al., 1998; Mitchell et al., 1995; Abe et al., 1998). Virus isolation until recently was considered the gold standard for the proving the etiology of viral retinitis, any viral infection. This requires the facility of specialized tissue culture laboratory, which is expensive. As described by us earlier the conventional method of viral isolation is time consuming and has low sensitivity compared with the molecular methods (Madhavan et al., 1999). Although theoretically a single viral particle is enough for virus isolation, the infectious titre is markedly reduced during transportation and storage resulting in false negativity by the conventional diagnosis (Mitchell et al., 1995). PCR is a rapid and reliable diagnostic tool for the detection of ocular infections. Many studies undertaken to prove the usefulness of PCR have concluded that PCR techniques are useful as rapid and sensitive adjuncts to clinical diagnosis (Knox et al., 1998). As seen clearly from this study there were no virus isolations from all the 21 intraocular fluids tested in spite of the immediate processing of the clinical sample. This failure to isolate the virus could be attributed to the limited amount of the clinical sample available. The results of uPCR targeting the DNA polymerase gene of HSV detected only 19% positivity

in the twenty-one tested. We had earlier shown that the sensitivity of the uPCR is only 0.5 femtograms for HSV 1 and 0.2 femtograms for HSV 2 (Madhavan et al., 1999), which is very low for the detection of the viral DNA, and therefore it is possible that negative results are likely. Since the uPCR detected both HSV 1 and 2 serotypes the amplified product need to be further processed to type them, serotyping the HSV strains is needed because of the frequency of reactivation of the disease which varied between HSV-1 and 2 (Cunningham et al., 1999). Emmett Cunningham *et al* studied on 2 cases of HSV associated retinitis who had earlier HSV associated encephalitis and later had a reactivation of HSV and developed PORN. They explained that HSV 1 affected primarily the retinal arterioles, whereas the serotype 2 affected the retinal veins more than the arterioles, which later leads to retinal detachment (Cuningham et al., 1996). Other conventional method like the neutralization test necessitates the virus isolation. PCR based Restriction fragment length polymorphism (PCR-RFLP) requires trained skilled personnel. DNA sequencing used for serotyping is an expensive procedure. Hence, in this study to aid the rapid detection as well as serotyping a seminested PCR for glycoprotein D gene was standardized and applied onto the intraocular clinical specimens. The usefulness of the glycoprotein D gene for HSV detection has been well established by many authors (Wang et al., 1999; Kudelova et al., 1995). The sensitivity of the snPCR was very high requiring only 0.02 attograms of the genome and this was further indicated by the seven positives picked

up by the snPCR, including the three missed by the uPCR. The specificity of the snPCR tested on the clinical isolates concordantly differentiated the HSV 1 and HSV 2 respectively with our earlier typing results indicating 100% specificity of the snPCR. The DNA sequencing also proved 100% homology with the standard strains of HSV 1 and 2. The result of the sequencing data was in concordance with that of the snPCR, indicating that the PCR can be used for serotyping. The dual specimens obtained from 2 cases of ARN also proved the specificity of the PCR as the same serotype, which was detected in AH, was later detected in VA of the same patient. Out of the two cases with dual specimens of AH and VA, HSV 1 DNA was detected in 1 case and HSV 2 detected in other case indicating that either of serotypes can cause the infection. Serotyping along with detection could be completed in 6-8 hours as against the 24-72 hours required for other conventional methods. As viral retinitis is caused by any of the three herpes viruses the intraocular fluids were also tested for the presence of DNAs of CMV and VZV. HSV is followed by VZV with a high positivity of 23.8% in cases of ARN, as VZV shares several biological features with well-characterized HSV (Abe et al., 1998). CMV DNA was detected in only 9.5 % i. e. only in 2 patients one in accordance with CMV retinitis as diagnosed clinically and another case of ARN. Isolated cases of ARN being caused by CMV have been reported and thus it is possible that CMV could be the etiological agent in this case also. Hence all the three herpes viruses have been detected indicating that any

of the three viruses can cause the infection leading to ARN, PORN, viral retinitis. Many authors have documented the usefulness of PCR for the laboratory diagnosis of CNS disease due to HSV. The assay is rapid, sensitive and specific enough to be used as a frontline test for the detection of HSV DNA, thus avoiding invasive and expensive brain biopsy specimen procedures which have been previously used as a benchmark for the laboratory diagnosis of HSV encephalitis. CSF specimens from patients without CNS infections do not commonly have detectable virus nucleic acids present in the fluid (Aslanzadeh et al., 1992; Rose et al., 1992). In addition, HSV DNA can be detected a early as 1 day after onset of clinical disease. The persistence of target DNA for an average of 14 days (range of 1 to 30 days) in CSF enhances the diagnostic sensitivity of this assay compared to that of HSV antigen or cell culture recovery of the virus, which have narrower diagnostic window (Gnffbnd et al., 1994; Ho et al., 1985; Kimura et al., 1991). 6.0% of the CSF specimens were positive for HSV 2 serotype and in two cases HSV was detected in both blood and the CSF samples. The clinical data was not available for any of the CSF samples. Many authors have documented that HSV2 and VZV are common causes of aseptic meningitis in adults even without rash (Davies et al., 2005; Bergstorm et al., 1996; Schlesinger et al., 1995). Even in our study we have a high incidence of HSV 2 when compared to HSV1 (2.7%). The restriction enzyme digestion of the snPCR amplified products also confirmed the serotype specificity of HSV. It is concluded, that the

standardized snPCR can be applied directly onto the clinical specimens for rapid detection and serotyping of HSV.

Epidemics of AHC are known to occur in the seasonal hot and humid conditions, which occur during the months of August and September in India (Christopher et al., 1977). The clinical features observed in the 157 patients were characteristic of AHC. All the specimens were initially tested by PCR for the presence of Ad DNA. The epidemic started with Ad and the serotype identity was established by molecular Serotyping. The Molecular serotyping method could be a good alternative to the 'gold standard' cell culture – neutralization method in view of its easy accessibility and expected sensitivity and the good correlation of the results with those obtained by the conventional methods (Ishii et al., 1987, Harrington et al., 2003; Bourlet et al., 2002). Studies by Takeuchi et al., have shown that the results obtained by the PCR sequence method coincided with the results of the conventional culture isolation – NT and it was demonstrated that the PCR sequence method is useful as a method of serotype identification. Hence PCR followed by sequencing of the amplicons was carried out and Ad type 8 was found to associated with the epidemic. In 15% of the patients the causative etiology was established to be Ad and of these, 66.6 % was isolated in Hep - 2 culture. DNA sequencing of these culture isolates also proved the serotype specificity as Ad8, as 98% homology was obtained with the same. We had earlier studied the epidemic that occurred in August of 2002 by PCR based

RFLP, which took at least 3 days, whereas by this method of molecular serotyping both detection and identification of the serotype could be obtained within 48 hrs. The specimens tested after October 10, 2006 were negative for Ad, and the clinical picture was suggesting a viral etiology, this provoked us to test for the other common viral etiologic agents of AHC namely Enteroviruses. One is EV70, which appeared form the first time around 1970 in West Africa and subsequently spread to the whole world (Kona et al., 1975). The other is Coxsackie A24 variant an antigenic variant of coxsackie A24, which was isolated for the first time in 1970 in Singapore (Murphy et al., 1972). We used the nested Pan enterovirus primer sequences for rapid screening of the samples for the identification of the etiologic agent. 77.0% of the patients were positive for EVs. These positive specimens were simultaneously tested using uniplex primers specific for VP1 region of CA24v. Only 49% of the patients turned positive for CA24v using these primers. The etiology of the remaining patients was studied by the molecular method of sequencing the PCR amplicons obtained in the Pan enterovirus PCR. 98% homology was obtained with that of the CA24v. The main drawback of the study is that the etiological agent of 8% of the patients could not be established, as they were negative for Ad and pan enterovirus. This could be explained due to the storage conditions, laboratory manipulations of several thawing – freezing process that resulted in the degradation of the EV RNA. The results of our study are in concordance with CDC report on outbreak of

conjunctivitis in Puerto Rico in 2004. Our EV isolation rate using vero cell cultures is 20.6 %, where Kishore et al., 2002 has obtained the same using HeLa cell cultures in contrast to our report of 57.4 using HeLa. The isolation rate using Hep - 2 cell cultures (36.7%) is in between vero and HeLa. Many authors have used different cell cultures like RD 19S cell line (45.6%) Kishore et al., 2002; Buffalo Green Monkey Kidney cells (28.9%) Broor et al., 1992; human embryonic kidney cells (30%) Gogate et al., 1979; HeLa (80%) Ponnuraj et al., 1986 for the isolation of EVs. Failure to isolate CA24v from other CS specimens could be attributed to low viral load, which is proved by only 49% positivity with uniplex PCR.

Hence to conclude the epidemic in June – September 2006 was a mixed epidemic with initially starting with Adenovirus and later replaced by the more hemorrhagic CA24v. Molecular techniques should be employed for the rapid identification of the etiological agent of the AHC epidemics and further confirmation of the serotype specificity can be established by nucleotide sequencing as reported by Oberste et al., 1999. Hence newer serotypes namely HSV2 and RGI are the main etiological agents in the causation of the disease and Coxsackie A24variant has caused the epidemic of acute hemorrhagic conjunctivitis in the south Indian population during June to September 2006.

SUMMARY AND CONCLUSIONS

Nucleic acid based molecular biological methods were applied on various clinical specimens for the detection of various viral etiological agents causing congenital cataract and epidemic conjunctivitis. Quantification of the Herpes simplex viral particles was done by the application of standardized Real Time PCR. The genotype identity was established by DNA sequencing. The serotype of HSV was confirmed using various techniques like PCR based Restriction Fragment Length Polymorphism (PCR - RFLP) and DNA sequencing. Conventional methods of Indirect Immunofluorescence staining (IIF) and virus isolation in cell cultures were also attempted. The present study indicated the association of Rubella virus (RV) with 12% of congenital cataract patients in less than one-year age. Twenty RV isolates have been obtained using the conventional cell culture methods. 18 of the twenty isolates have been genotyped as belonging to RGI and two to RGII. Direct evidences of association of RV and HSV 2 were demonstrated in 18 (36 %) lens aspirates with RV in 9 (18 %) and HSV 2 in 9 (18 %) others. VZV was not detected in any of the lens aspirates tested. The results of PCR-RFLP and DNA sequencing for HSV serotype confirmation were in concordance with that of the snPCR. Conjunctival swabs collected from patients of Epidemic conjunctivitis indicated the presence of Adenovirus (Ad)

in 15% of the patients and remaining 77.0% of the patients were positive for Enterovirus (EV). 49% of these patients turned positive for Coxsackie A24 variant (CA24v) and DNA sequencing indicated 98% homology with that of the CA24v.

Conclusion:

For the first time in India, such direct evidences of these virus associations with congenital cataract have been demonstrated. HSV 2 could also play a major etiological role in the causation of congenital ctaract. Hence newer serotypes namely HSV2 and RGI are the main etiological agents in the causation of the disease in Indian Population and Coxsackie A24variant has caused the epidemic of acute hemorrhagic conjunctivitis in the south Indian population during June to September 2006.

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PUBLICATIONS:

Shyamala G., Sowmya. P, Sudha. B, Malathi. J. Lily Therese K, Madhavan H.N. Application of Polymerase chain reaction to differentiate herpes Simplex Virus 1&2 serotypes in culture Negative intraocular aspirates. Indian J of Medical Microbiology. 2005 October; 23(4): 239 - 45.

Shyamala. G, Malathi. J, Samson Moses. Y, Lily Therese. K, Madhavan HN. Application of Nested Reverse Transcription Polymerase chain reaction for the detection of Rubella virus in clinical specimens. *Accepted for publication in Indian Journal of Medical Research.*

ORAL PRESENTATIONS:

Shyamala G, Malathi J, Madhavan HN. "Genotyping of Rubella virus isolates" presented in XXXth National congress of Indian association of Medical Microbiologists held at Nagpur on 27th-29th October 2006.

Shyamala G, Sowmya P, Malathi J, Therese KL, Madhavan HN. "Determination of viral etiology in congenital cataract by nucleic acid based amplification techniques" presented in the free paper session in CME on ocular Microbiology held at Madurai on 5th August 2006.

Shyamala. G, Malathi. J, Madhavan HN, Lily Therese. K. "Application of Reverse transcriptase polymerase chain reaction (RT-PCR) for the detection of Rubella virus associated congenital cataract presented in

IERG conference held at L.V. Prasad eye institute on 30 & 31st of July 2005.

Dr.H.N. Madhavan, **Shyamala. G**, Malathi.J, Lily Therese.K "Application of semi – nested Polymerase chain reaction for the detection of herpes simplex virus serotypes in clinical specimens" presented in XXVIIIth National congress of Indian association of Medical Microbiologists held at Lucknow on the 21st and 24th September 2004.

LIST OF POSTER PRESENTATIONS:

Presented poster in the "Exclusive Meet on Ophthalmic Research" "Optimization of Nucleic acid amplification techniques by Polymerase chain reaction for differentiation of Herpes simplex virus serotypes 1&2 in ocular specimens"– Indian eye Research group, 20th - 22nd August 2004.

"Standardization of semi- nested polymerase chain reaction for the detection of Herpes simplex virus serotypes 1 & 2 in clinical specimens"chapter meeting in Coimbatore, October 2004.